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ORIGINAL ARTICLE

Validating the Genus *Pocheina* (Acrasidae, Heterolobosea, Discoba) Leads to the Recognition of Three Major Lineages Within Acrasidae

Alexander K. Tice[1,2] | Kevin Regis[3] | Timothy E. Shutt[4] | Frederick W. Spiegel[3] | Matthew W. Brown[1,5] | Jeffrey D. Silberman[3,6]

[1]Department of Biological Sciences, Mississippi State University, Mississippi State, Mississippi, USA | [2]Department of Biological Sciences, Texas Tech University, Lubbock, Texas, USA | [3]Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, USA | [4]Department of Biochemistry & Molecular Biology, University of Calgary, Calgary, Canada | [5]Institute for Genomics, Biocomputing & Biotechnology, Mississippi State University, Mississippi State, Mississippi, USA | [6]Biology Center CAS, Institute of Parasitology, České Budějovice, Czech Republic

Correspondence: Matthew W. Brown (matthew.brown@msstate.edu) | Jeffrey D. Silberman (jeff.silberman@paru.cas.cz)

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ABSTRACT

Pocheina and *Acrasis* are two genera of heterolobosean sorocarpic amoebae within Acrasidae that have historically been considered close relatives. The two genera were differentiated based on their differing fruiting body morphologies. The validity of this taxonomic distinction was challenged when a SSU rRNA phylogenetic study placed an isolate morphologically identified as "*Pocheina*" *rosea* within a clade of *Acrasis rosea* isolates. The authors speculated that pocheinoid fruiting body morphology might be the result of aberrant *Ac. rosea* fruiting body development, which, if true, would nullify this taxonomic distinction between genera. To clarify Acrasidae systematics, we analyzed SSU rRNA and ITS region sequences from multiple isolates of *Pocheina*, *Acrasis*, and *Allovahlkampfia* generated by Polymerase Chain Reaction (PCR) and transcriptomics. We demonstrate that the initial SSU sequence attributed to "*P. rosea*" originated from an *Ac. rosea* DNA contamination in its amplification reaction. Our analyses, based on morphology, SSU and 5.8S rRNA gene phylogenies, as well as comparative analyses of ITS1 and ITS2 sequences, resolve Acrasidae into three major lineages: *Allovahlkampfia* and the strongly supported clades comprising *Pocheina* and *Acrasis*. We confirm that the latter two genera can be identified by their fruiting body morphologies.

1 | Introduction

In 1873, Cienkowski described a microorganism he found on collections of dead lichenized wood in Russia (Cienkowski 1873). Its fruiting body (sorocarp) was pink in color with a stalk consisting of a row of wedge-shaped cells supporting a globular mass of spores at its apex. Each spore was said to contain pinkish cytoplasm and a nucleus, and when spores germinated, a limax-shaped amoeba with pink cytoplasm emerged. Cienkowski's description of "*Guttulina rosea*" was the first of a non-dictyostelid sorocarpic amoeba (cellular slime mold) (Cienkowski 1873). Aside from transferring the organism to the newly erected genus *Pocheina* due to the recognition that the genus name *Guttulina* was already in use (Loeblich Jr. and Tappan 1961), no work was done on the organism until its rediscovery in the 1970s (Raper 1973). A second species of *Pocheina* was later described, *P. flagellata*; because anteriorly biflagellated cells as well as limax-shaped amoebae emerged upon spore germination (Olive et al. 1983).

Another sorocarpic amoeba was discovered by van Tieghem (1880), named *Acrasis granulata*. It was found fruiting on spent beer yeast as columnar rows of spores, brownish in color, that hatched amoeboid cells. Eighty years later, Olive and Stoianovitch (1960) added a new species to the genus *Acrasis*, *Ac. rosea*, because it matched the unillustrated text description of *Ac. granulata*. *Acrasis rosea* was found fruiting on collections of leaves and inflorescences of *Phragmites* sp. grass, and its spores germinated to produce limax-shaped amoebae with pinkish-orange cytoplasm (Olive and Stoianovitch 1960). The fruiting bodies of *Acrasis* differed from those of *Pocheina* in that they formed chains of spores rather than a globose mass at the apex of the stalk cells (Olive and Stoianovitch 1960).

Olive et al. (1983) first proposed that *Pocheina* and *Acrasis* were closely related. Subsequently, they were placed with the vahlkampfiid amoebae into Heterolobosea (Page and Blanton 1985) because of the eruptive motion of the pseudopodia during locomotion of the amoeboid trophic cells, similarities in mitochondrial cristae structure (flattened discoidal cristae), and the close association of the mitochondria and endoplasmic reticulum (Dykstra 1977; Olive 1975; Page and Blanton 1985; Pánek et al. 2017). Despite these morphological and ultrastructural similarities, *Pocheina* and *Acrasis* were always maintained as separate genera based primarily on sorocarp morphology (Dykstra 1977; Page and Blanton 1985), but the exact relationship between *Acrasis* and *Pocheina* remained unclear.

In the first molecular phylogenetic study to include numerous geographically distributed isolates of '*Ac. rosea*', it was shown that what was once thought to be merely morphological plasticity in the fruiting bodies among different isolates were phylogenetically significant characteristics that could be used in conjunction with molecular data to delineate species (Brown, Silberman, et al. 2012). Based on the congruence of morphology and molecular phylogenetic data using the nuclear-encoded SSU rRNA gene (SSU) sequence, at least four distinct species of *Acrasis* exist (Brown, Silberman, et al. 2012). Included in this study was a partial SSU sequence generated from uncultured fruiting bodies, each topped with a globular spore mass, picked directly from its natural substrate, that is, the morphotype typical of *Pocheina*. Surprisingly, this putative *Pocheina* ("*P. rosea*") sequence was nested in a clade that contained all verified isolates of *Ac. rosea* (Brown, Silberman, et al. 2012). This led the authors to postulate that slight alterations during the development of *Ac. rosea* may be responsible for the formation of the chainless sorocarps (Brown, Silberman, et al. 2012). This hypothesis was supported by the observation that long-term cultured isolates of *Ac. rosea* and *Ac. helenhemmesae* occasionally produced sorocarps with a globose spore mass atop a cellular stalk (Brown et al. 2010; Brown, Silberman, et al. 2012). If true, then the morphological difference ascribed to the fruiting bodies of *Acrasis*, and especially *Pocheina*, would be taxonomically uninformative. Although the phylogenetic results were interpreted as best as possible with the available data, for a variety of reasons, we were suspicious of the "*Pocheina*" isolate's position within *Acrasis* because slight variations in sorocarp morphology among species of *Acrasis* were representative of a large amount of molecular divergence in the SSU sequence among the different species, and the sorocarp morphology in previous cultures of the two known species of *Pocheina* (*P. rosea* and *P. flagellata*; Cienkowski 1873; Olive et al. 1983; Raper 1973) remained stable through passaging. No culture of either species of *Pocheina* has been known to produce sorocarps that resemble sorocarps of any of the known species of *Acrasis* (Olive and Stoianovitch 1960; Olive et al. 1983; Raper 1973). Thus, the position of '*P. rosea*' in the SSU phylogeny calls into question the foundation of separating the genera *Acrasis* and *Pocheina* based on fruiting body morphologies, and the validity of the genus *Pocheina*.

To clarify the systematics of the genus *Pocheina* and the relationship of *Pocheina* spp. to *Acrasis* spp., we collected additional strains of both *P. rosea* and *P. flagellata* from widely separated geographic locales and sequenced their SSU and/or ITS regions (ITS1, 5.8S rRNA gene, ITS2) for comparative analyses. Included in the analyses were newly sequenced ITS regions from all isolates of *Acrasis* spp. studied in Brown, Silberman, et al. (2012). The generation of ITS sequences from these morphologically/phylogenetically delineated *Acrasis* spp. further resolved the relationship between *Acrasis* and *Pocheina* spp. Additionally, these data provided an ideal set of "good" species to assess the benchmark hypotheses generated for *Naegleria* and closely related heterolobosean, which posited that each species possesses unique ITS sequences and that each genus forms a distinct clade in 5.8S trees (De Jonckheere 1998, 2004; De Jonckheere and Brown 2005). Our results demonstrate that (1) sorocarp morphology correlates with molecular phylogenetic inference, (2) the "*P. rosea*" SSU sequence reported in Brown, Silberman, et al. (2012) is a contamination from an *Ac. rosea* isolate, (3) the first publicly available molecular data from *Pocheina* spp. are reported, (4) all isolates identified as *Pocheina* spp. form a monophyletic group separate from *Acrasis* spp., which (5) is also monophyletic, and finally, (6) these data are the basis of systematic revisions that establish the monophyly of each major lineage within Acrasidae (including *Allovahlkampfia*).

2 | Materials & Methods

2.1 | Bark Sampling and Morphological Observation

Bark from *Pinus* spp. trees was collected at chest height from five different sites, including one site (HUNT, yielding isolates HUNT 1 and 2) that was sampled on two separate occasions 5 years apart (Table 1). Bark samples were placed into paper bags, brought back to the laboratory, cut into small <1 cm pieces, and placed on sterile weak malt yeast agar (wMY) (0.75 g K_2HPO_4, 0.002 g yeast extract, 0.002 g malt extract, 15.0 g agar/liter DI H_2O) Petri plates and hydrated with a drop of sterile DI H_2O. Plates were incubated at room temperature (ca. 22°C) under normal ambient light conditions of the laboratory. After 2–7 days, the pieces of bark were scanned for bright pink pocheinoid fruiting bodies using a Leica M205

TABLE 1 | *Pocheina* samples obtained in this study along with their locality, isolation substrate, morphology of cells that germinated from spores, and the fate of germinants on agar growth slides over time.

Species	Isolate	Collection site	GPS coordinates	Substrate	Germinant	Fate of germinant
P. rosea	HI12	Hilo, HI, USA	N 19 39′20″ W 155 4′31″	Bark of *Pinus radiata*	Amoeba	Cyst
P. rosea	NJ13	Wall Township, NJ, USA	N 40 10′6″ W 74 6′10″	Bark of *Pinus* sp.	Amoeba	Death
P. flagellata	HUNT1	Huntsville, AR, USA	N 36 2′21″ W 93 40′46″	Bark of *Pinus* sp.	Flagellate	Amoebae or death
P. flagellata	HUNT2	Huntsville, AR, USA	N 36 2′21″ W 93 40′46″	Bark of *Pinus* sp.	Flagellate	Death
P. flagellata	GERM14	Schwarzenbruck, Germany	N 49 21′21″ W 11 13′25″	Bark of *Pinus* sp.	Flagellate	Cyst or death
Pocheina sp.	LW14	Fayetteville, AR, USA	N 36 5′36″ W 94 21′51″	Bark of *Pinus* sp.	Unknown	N/A

dissecting microscope (Wetzlar, Germany) with reflected light. Images of fruiting bodies were taken with an attached Canon 650D (Tokyo, Japan) digital camera under reflected light or an Axioskop 2 Plus (Zeiss, Berlin, Germany) at 10× with an attached Canon 650D camera under transmitted light. To observe spore germination, culture slides were created by melting a ~4 mm × 4 mm block of lactic acid adjusted wMY agar at pH ~ 5 (as described below) between a slide and cover glass. After cooling, the cover glass was removed, leaving a thin square of solidified agar. A single fruiting body was removed from the bark with a 0.15 mm Austerlitz Insect Pin (Carolina Biological, Burlington, NC, USA) and placed onto the culture slides along with a drop of DI H_2O (Brown, Silberman, et al. 2012; Spiegel et al. 2005). Spore germination and trophic cells were observed using an Axioskop 2 Plus light microscope equipped with 40× and 63× objectives using both phase contrast and differential interference contrast (DIC) microscopy. Photomicrographs of these cells were acquired using a Canon Rebel T2i, Canon 650D, or Canon 5DS digital camera. Attempts to culture *Pocheina* were made by streaking spores onto wMY agar plates adjusted to pH ~5 by adding 3 drops of 5% lactic acid during pouring (Olive et al. 1983), with either an unidentified species of *Aureobasidium*, *Rhodotorula mucilaginosa*, or *Escherichia coli*.

Allovahlkampfia ("*Solumitrus*") *palustris* (PRA325) *sensu* Gao et al. (2022) was purchased from the American Type Culture Collection (ATCC). *Allovahlkampfia* sp. strains BA and OSA were isolated as amoebae from the bark of a downed maple tree log in Halifax, Nova Scotia, Canada (44 38′06″ N, 63 32′21″ W) and from the fruiting body of a basidiomycete jelly fungus, *Dacrymyces* sp., that was growing on an unidentified rotting log in Kejimikujik National Park, Nova Scotia, Canada (44 25′03″ N, 65 17′54″ W), respectively (Shutt 2006). Each of these allovahlkampfiids was propagated in either liquid wMY or hay-infusion medium (ATCC 802) in tissue culture flasks supplemented with *E. coli* as the food source.

2.2 | Genomic DNA Extraction

Genomic DNAs from the *Acrasis* taxa used to amplify the ITS region were from the study of Brown, Silberman, et al. (2012). From new *Pocheina* isolates, two to three sorocarps immediately surrounding the sorocarp taken to observe spore germination were used for DNA extraction. These sorocarps were picked directly from the primary bark substrate using an ethanol flame-sterilized Austerlitz Insect Pin and placed into 30 μL of Epicenter QuickExtract DNA extraction solution. Aside from the modified solution volume, DNA was liberated from spores using the manufacturer's recommended protocol. Genomic DNA from *Al. palustris*, *Allovahlkampfia* strain BA and strain OSA was isolated from cell pellets using the Gentra Puregene Tissue Kit (Qiagen) following the manufacture's protocol.

2.3 | Polymerase Chain Reaction (PCR) From gDNAs

The ITS region (contiguous 3′ end of SSU, ITS1, 5.8S, ITS2, 5′ end of large subunit (LSU) rRNA gene) and SSU genes were PCR amplified in 25 μL total reaction volumes using Q5 High-Fidelity DNA Polymerase (2× master mix, New England Biolabs, Ipswich, MA, USA) for 30 cycles with combinations of "universal" eukaryotic primers (De Jonckheere and Brown 2005; Medlin et al. 1988) and custom primers designed against *Allovahlkampfia* spp. and *Acrasis* spp. SSU and ITS sequences (Tables 2–4). For each PCR, elongation times were based on slight overestimates of the expected amplicon size, and annealing temperatures were specified using NEB's Tm calculator. Post-cycling, 20 μL of each PCR reaction was electrophoresed on a 1% agarose gel in TA buffer (9.68 g Tris Base, 2.28 mL glacial acetic acid/liter DI H_2O) containing SybrSafe (Life Technologies, Grand Island, NY). If weak or no amplicon was seen on the gel, 1 μL of the primary PCR was utilized for nested or semi-nested PCR (Tables 2 and 3). Upon

TABLE 2 | PCR amplification and product information for the nuclear-encoded SSU of *Pocheina* spp. and *Allovahlkampfia palustris* amplified in this study.

Isolate	Species	1° PCR primers	2° PCR primers
HI12	*P. rosea*	Acd41F: Medlin B	Acd54F: Acd687R, 300F: Acd766R, Acd720F: Acd1424R
NJ13	*P. rosea*	Acd41F: Medlin B	Acd49F: Acd687R, Acd645F: Allo766R, Acd720F: Acd1425R, Acd1380F: Allo1460R, Allo41F: Allo552R
HUNT1	*P. flagellata*	Acd41F: Medlin B	Acd54F: Allo1460R
PRA-325	*Al. palustris*	MedlinA: 1492R	

TABLE 3 | PCR amplification and product information for the ITS region of all isolates of *Pocheina*, *Acrasis*, and *Allovahlkampfia* amplified in this study.

Isolate	Species	1° PCR primers	2° PCR primers	3° PCR primers
Acr_1Ba5-2	*Ac. helenhemmesae*	1350F: 60R	1495F: 60R	—
Acr_AusBG-8-1	*Ac. helenhemmesae*	1350F: 60R	1400F: 60R	1495F: 60R
Acr_BM07-A1-1	*Ac. helenhemmesae*	1350F: 60R	1495F: 60R	—
Acr_HI06-7a-5a	*Ac. helenhemmesae*	1350F: 60R	1495F: 60R	—
Acr_HI09-40b-1	*Ac. helenhemmesae*	1350F: 60R	1495F: 60R	—
Acr_HI06-7a-5b	*Ac. kona*	1350F: JITSR	—	—
Acr_MYA-3364	*Ac. kona*	1350F: 60R	1495F: 60R	—
Acr_MYA-3509	*Ac. kona*	1400F: JITSR	—	—
Acr_T-235	*Ac. kona*	1350F: 60R	—	—
Acr_1Ba-5-1	*Ac. rosea*	1350F: JITSR	—	—
Acr_NJ08-1A-1	*Ac. rosea*	1350F: 60R	1495F: 60R	
Acr_NZ05-36a-1	*Ac. rosea*	1400F: JITSR	1495F: 60R	—
Acr_UK05-8-2	*Ac. rosea*	1350F: 60R	1495F: 60R	—
Acr_LOST07L112	*Ac. rosea*	1350F: 60R	1400F: 60R	1495F: 60R
Acr_THAI-08-23-1	*Ac. takarsan*	1350F: JITSR	—	—
HI12	*P. rosea*	1350F: 60R	1400F: 60R	—
NJ13	*P. rosea*	1350F: 60R	1400F: 60R	—
HUNT1	*P. flagellata*	1350F: 60R	1400F: 60R	—
GERM14	*P. flagellata*	1350F: 60R	1400F: 60R	—
LW14	*Pocheina* sp.	1350F: 60R	1400F: 60R	—
BA	*Allovahlkamphia* sp.	1350F: 60R	—	—
OSA	*Allovahlkampfia* sp.	1350F: 60R	—	—

strong amplification, the DNA bands were cut out of the gel with a razor blade and purified by centrifugation through a 200-μL barrier pipette tip as described in Becker et al. (2024). The ITS region was amplified from two *Allovahlkampfia* strains (BA, OSA), five new *Pocheina* isolates (HUNT1, LW14, NJ13, HI12, and GERM14) as well as from LOST07L112 and each *Acrasis* spp. from the DNAs isolated by Brown, Silberman, et al. (2012) (Table 3). Also amplified were the nearly complete SSU genes of three new *Pocheina* isolates (HUNT2, NJ13, and HI12) and *Al. palustris* (Table 2).

In nearly all instances, PCR products were sequenced directly. In a few cases, weakly amplified amplicons were cloned using the TOPO-Blunt Cloning Kit for Sequencing (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. Recombinant plasmids were isolated using the Zyppy Plasmid Miniprep Kit

TABLE 4 | Primer names and sequences used for the amplification of the nuclear-encoded SSU and ITS regions of all isolates of *Pocheina*, *Acrasis*, and *Allovahlkampfia* amplified in this study.

Primer name	Sequence	Target	Direction
MedlinA[a]	5′-CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT-3′	SSU	Forward
Acd41F	5′-ATATGCTTGTCTCAAAGACTAAGC-3′		
Acd49F	5′-GTYTYAAAGAYTAAGCCATGCA-3′	SSU	Forward
Acd54F	5′-AAAGAYTAAGCCATGCACATG-3′	SSU	Forward
Allo552R	5′-CAACTTMAGCTGATAGATAAG-3′	SSU	Reverse
Acd645F	5′-ATRGTTTGGAATGRKTTTAGATT-3′	SSU	Forward
Acd687R	5′-CACCAGACTHTYCCTYTAGTC-3′	SSU	Reverse
Acd720F	5′-GTAATTCCAGCTCTAGWAGYGTAT-3′	SSU	Forward
Allo766R	5′-CTTRGGTCAACTACGAGCG-3′	SSU	Reverse
Acr1300F	5′-TACTACACTRTTRATACT-3′	SSU	Forward
Acr1350F	5′-CATTAAYGTGACRGGGATAGCTG-3′	SSU	Forward
Acd1380F	5′-TAGTCGCAAGGCCGAAACTTA-3′	SSU	Forward
1400F[a]	5′-TTGTACACACCGCCCGTCGC-3′	SSU	Forward
Acd1424R	5′-CCGCAAACTCCACTCCTGG-3′	SSU	Reverse
Allo1460R	5′-AAGGTTCAGTTAATTTCCCCA-3′	SSU	Reverse
Acr1495F	5′-GAGAAGTCGTAACAAGGTCT-3′	SSU	Forward
1492R[a]	5′-ACCTTGTTACGACTT-3′	SSU	Reverse
MedlinB[a]	5′-CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC-3′	SSU	Reverse
Acr5.8F	5′-GTAATGTGAATCGCAACTAAC-3′	5.8S	Forward
Acr5.8R	5′-GTTAGTTGCGATTCACATTAC-3′	5.8S	Reverse
LSU60R	5′-TCCTCCVCTTAKTRATATGCTTA-3′	LSU	Reverse
JITSR[b]	5′-CCGCTTACTGATATGCTT-3′	LSU	Reverse
JITSRb	5′-CTYTTCVCTCGCMGKTAC-3′	LSU	Reverse

[a]From or modified from Medlin et al. (1988).
[b]From De Jonckheere and Brown (2005).

(Zymo Research, Irvine, California, USA) following the manufacturer's protocol. Samples were Sanger sequenced on an Applied Biosystems 3130xl Genetic Analyzer. Both the SSU and ITS regions were sequenced completely in both directions. All sequences were edited and assembled using Sequencher v5.1 (GeneCodes, Ann Arbor, MI, USA). No mixed peaks were seen on the chromatograms for any of our sequences, indicating that no microheterogeneity in the SSU or the ITS region exists within or among the cells of any of our isolates.

2.4 | Ultra-Low Input Transcriptomics

For *Pocheina* isolate HUNT2, we employed an ultra-low input RNA-Seq approach to obtain the SSU and ITS region sequences. A single sorocarp (~20 cells) was picked from the bark substrate with a 30-gauge platinum wire and placed directly into a 200-μL thin-walled PCR tube. The cells were subjected to a modified version of Smart-Seq2 mRNA extraction and cDNA library preparation (Picelli et al. 2014) that included an additional freeze thaw step for cell lysis, as described in Onsbring et al. (2020). The resulting cDNA library was prepared for sequencing on an Illumina platform using the Nextera XT DNA Library Prep Kit (Illumina, CA, USA) following the manufacturer's protocol with dual index primers. The library was sequenced using an Illumina HiSeq 4000 at Genome Quebec (Montreal, Canada).

2.5 | Transcriptomic Assembly and Bioinformatic Retrieval of the SSU and ITS Regions

Low-quality bases, adaptor sequences, and Smart-Seq2 primer sites were removed from the HUNT2 raw sequencing read files using TRIMMOMATIC v0.35 (Bolger et al. 2014). The surviving reads were assembled using the de novo assembly program TRINITY v2.1.1 (Grabherr et al. 2011). The full length SSU and ITS region were retrieved using BLASTN (Altschul et al. 1990)

and Acrasidae SSU and ITS data to query the transcriptome assembly.

2.6 | Phylogenetic and Comparative Sequence Analyses

Phylogenetic trees were inferred from 43 SSU rRNA gene sequences, including our new Allovahlkampfiid and *Pocheina* sequences, along with other Acrasidae and representative outgroup heteroloboseans (*Naegleria*, *Willaertia*, *Pleurostomum*, and *Tulamoeba* spp.). Trees were inferred from an inclusion set of 1868 unambiguously aligned nucleotide positions using maximum likelihood (ML) and Bayesian inference (BI) methods. Alignments were inferred with MAFFT-LINSI v7.407 (Katoh and Standley 2013) with default parameters by using the add function, adding new sequences to a seed alignment from Brown, Silberman, et al. (2012). Uninformative sites were removed using BMGE v1.12 (Criscuolo and Gribaldo 2010) with a maximum gap rate allowed per character set to 0.6 (-g 0.6). A general time reversible + gamma distribution (GTR + G) model of nucleotide change was implemented in RAxML v8.2.12 (Stamatakis 2014) using 25 discrete gamma rate categories. The best scoring ML tree of 300 independent "rapid-hill climbing" tree searches was mapped, with topological support assessed by ML analyses of 1000 nonparametric bootstrap replicates in RAxML under the same model. Bayesian analyses run in MrBayes v3.2.7 (Ronquist et al. 2012) consisted of two independent Markov chain Monte Carlo (MCMC) runs of 50,000,000 generations, printing trees every 1000 generations with a "burnin" of 7,676,000 generations, by which time all parameters converged, as assessed by an average standard split deviation (ASD) that plateaued at <0.003 and the potential scale reduction factor convergence diagnostic.

Our new ITS1, 5.8S gene, and ITS2 sequences of *Pocheina*, *Acrasis*, and allovahlkampfiids were included in pairwise sequence comparison, compositional, and phylogenetic analyses. Within Heterolobosea, only the 5.8S gene sequences could be confidently aligned and utilized for phylogenetic analyses. Forty-five 5.8S sequences, including those from each new *Acrasis* spp., *Allovahlkampfia* spp., and *Pocheina* spp., along with publicly available 5.8S sequences from other members of Acrasidae and representative outgroup sequences from *Naegleria* spp., were aligned using MAFFT-GINSI with default parameters. Sites not part of the 5.8S and those that were not confidently homologous were removed by hand in Aliview v1.26 (Larsson 2014). Maximum likelihood and Bayesian trees and support values were inferred as described for the SSU analyses, with the only difference being that the first 8,191,000 generations were discarded as burn-in in the Bayesian analysis.

To determine which *Allovahlkampfia* group our new *Allovahlkampfia* BA and OSA isolates belonged to, we conducted unrooted ML and Bayesian analyses of the entire ITS region of all *Allovahlkampfia* strains, as in Gao et al. (2022). All allovahlkampfiid ITS sequences were aligned using MAFFT-LINSI with default parameters. Uninformative sites were removed using BMGE v1.12 with a maximum gap rate allowed per character set of 0.6, resulting in 460 sites. Maximum likelihood and Bayesian trees and support values were inferred as described for the SSU analyses, with the only difference being that the Bayesian ASD plateaued at <0.002 and removed the first 6,651,000 generations as burn-in.

Finally, we generated a concatenated 5.8S and SSU dataset for phylogenetic analyses. We collected only Acrasidae sequences of both SSU and 5.8S genes with no outgroup taxa to increase the number of confidently aligned sites. Each gene was aligned using MAFFT-LINSI with default parameters. The SSU alignment was trimmed with BMGE with a maximum gap rate allowed per character set to 0.6. The 5.8S alignment was trimmed by hand. This resulted in 2013 bp from SSU and 170 bp from 5.8S. The SSU and 5.8S sequences were concatenated by hand when the data for both genes was available. In taxa where we only had one of the genes, the missing gene was treated as missing data. This resulted in a dataset of 41 taxa and 2173 nucleotide sites. Maximum likelihood and Bayesian trees and support values were inferred as described for the SSU analyses, with the only difference being that the Bayesian ASD plateaued at <0.002, and the first 3,716,000 generations were discarded as burn-in.

Uncorrected pairwise sequence differences (ignoring gaps) among the ITS1 and ITS2 were calculated between all Acrasidae genera, within each genus, and among species within a genus using the custom script (pdistcalculator.py, https://github.com/socialprotist/pdistcalculator.py/). The alignments of the ITS1 and ITS2 regions were individually analyzed. Sequence repeat regions were assessed by dot blots using the YASS genomic similarity search tool (Noe and Kucherov 2005) comparing each sequence to itself, accessed through the web portal, https://bioinfo.univ-lille.fr/yass/index.php, using default parameters.

3 | Results and Discussion

3.1 | Morphological Observations

Five new strains of *Pocheina* were collected for morphological and molecular analyses (Table 1). The morphology of sorocarps and trophic cells of all putative *Pocheina* spp. isolates was characteristic of the genus description (Olive et al. 1983). The fruiting bodies were pinkish orange in reflected light and were made up of a row or rows of wedge-shaped stalk cells topped by a globose mass of spores connected to one another by raised hila (Figure 1A–G). Slight variation in sorocarp size existed within and among isolates (Figure 1A–G). All attempts at *Pocheina* spore germination were successful on wMY agar (adjusted to pH ~5.0) except for LW14, which consistently failed to germinate. Using the interpretation of Olive et al. (1983), each isolate was assigned to a described species based on the morphology of trophozoites that emerged from spores (Table 1). Isolates were designated *P. flagellata* if a binucleated plasmodium (Figure 1J) that subsequently cleaved to become 2 uninucleate flagellates emerged from spores (Figure 1J–L). Isolates were assigned to *P. rosea* if a nonflagellate, uninucleate amoeboid cell emerged from spores (Figure 1M,N). Though variation was noted among the fruiting bodies seen on *Pinus* bark, we could not predict ahead of time if an amoeba or flagellate would germinate from

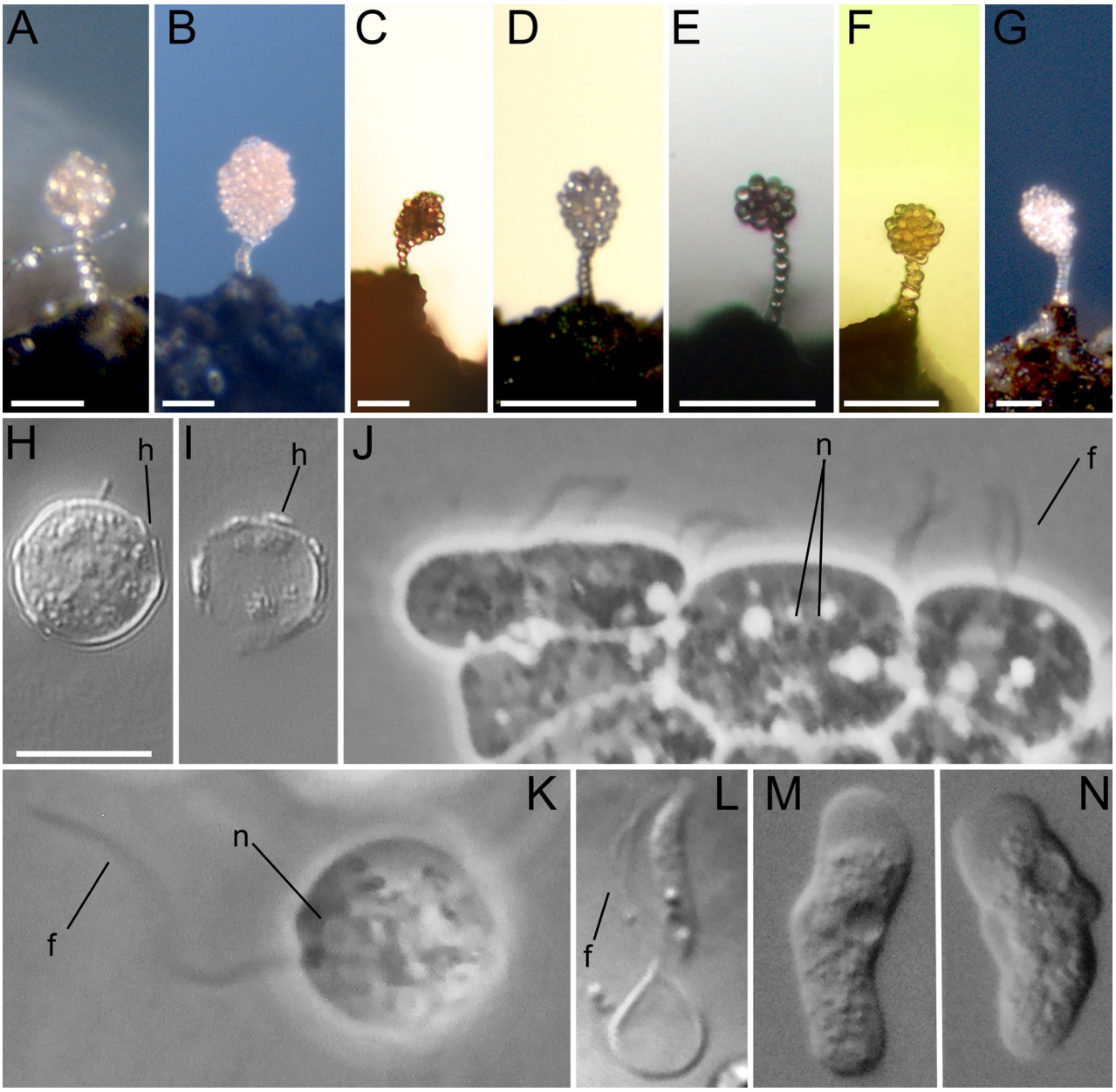

FIGURE 1 | Light microscopy images of *Pocheina* strains. Sorocarps of *Pocheina flagellata* strains GERM14 (A), HUNT1 (B), HUNT2 (C); *Pocheina rosea* isolates HI12 (D), NJ13 (E), LOST07L112 (F); (G) *Pocheina* sp. LW14. (A–G) Each scalebar = 50 μm. A,B,G are reflected light and C–F are transmitted light. (H) Spore of *P. rosea* LOST0711L2 with several visible raised hila (h). (I) Empty spore wall with raised hila (h). (J) Germinants from spores of *P. flagellata* strain HUNT1. The center cell is a binucleated amoebae before dividing into a single nucleated flagellate. Two flagella are annotated in the right most flagellate. (K) Swimming flagellate of *P. flagellata* HUNT1. (L) Elongated flagellate of *P. flagellata* GERM14, flagella (f). (M, N) Amoebae of *P. rosea* isolate NJ13. Image H–N are to scale, scalebar = 10 μm.

spores. Germinated trophic cells (either type) did not appear to divide but remained active for 1 h-4 days before the flagellates died, turned to amoebae, or the amoebae encysted (Table 1). Excystment did not occur under our culturing conditions, even when we passed cysts to fresh agar and food sources. Thus, trophic cells were never again observed after encystment and long-term cultures could not be established for any of the *Pocheina* strains, including the LOST07L112 isolate described in Brown, Silberman, et al. (2012).

The amoebae of *P. rosea* (isolates HI12 and NJ13) moved with eruptive pseudopodia, as did the amoebae that emerged from germinated spores of the LOST07L112 isolate (Brown, Silberman, et al. 2012). *Pocheina flagellata* cells (isolates HUNT1, HUNT2, GERM14) were semi-amoeboid when emerging from spores (Video S1). Once the morphology of flagellated trophozoites stabilized, they had a constant body shape and swam using their two anterior flagella (Figure 1K,L and Videos S1 and S2). As noted by Olive et al. (1983), flagellate morphology was stable within, but variable among isolates, which parallels our observations of the HUNT and GERM isolates. The flagellated cells of HUNT (1 and 2) were spherical to obovate in shape with a short yet distinct rostrum (Figure 1J). The flagellated cells of GERM14 were narrow, elongated, and tapered at the posterior end (Figure 1L). On agar

culture slides of HUNT1, which were kept for several days, flagellates transitioned into crawling nonflagellate amoeboid cells, as previously reported for *P. flagellata* (Olive et al. 1983). We did not directly observe the transition in real time, but saw that amoebae were present on older agar culture slides. This flagellate-to-amoeba transformation is associated with many heterolobosean (Pánek et al. 2017) and may be an ancestral trait for the entire lineage. Although the agar culture slides made photo-documentation difficult, videos captured the morphological essence of flagellated cells (Videos S1 and S2).

3.2 | Phylogenetic and Molecular Results

The nearly complete SSU gene for *P. rosea* isolates HI12 and NJ13 and *Al. palustris* was generated using a PCR approach, while the SSU from *P. flagellata* HUNT2 was bioinformatically recovered from its transcriptome, as well as the ITS region. Concurrently, we generated the complete nuclear-encoded ITS region (with the 3′ end of the SSU, ITS1, 5.8S, ITS2, 5′ end of the LSU) for six *Pocheina* isolates (five new strains plus LOST07L112), each of the *Acrasis* spp. isolates from Brown, Silberman, et al. (2012), and two *Allovahlkampfia* spp. through PCR amplification.

3.2.1 | Introns

Many Acrasidae and other heterolobosean taxa have group I introns within their SSU rRNA genes (Wikmark, et al. 2006; Brown, Silberman, et al. 2012), including two of our new *P. rosea* isolates (HI12 and NJ13, Figure 2). The naming system of Group I introns corresponds to their position in the *E. coli* SSU (16S, Johansen and Haugen 2001; GenBank accession AB035922). *Pocheina rosea* isolate HI12 minimally possesses three Group I introns that are located at sites known to be common intron sites (S516, S895, and S1199). These introns range from 264 to 1026 bp. We cannot be certain if this isolate's SSU has additional introns because we were not able to obtain its complete SSU and are missing a site that commonly possesses group I introns in other Heterolobosea (i.e., the gap in HI12's SSU sequence encompasses the 18 bp before and 11 bp after the S956 Group I insertion site, Figure 2). *Pocheina rosea* NJ13 has more Group I introns (five) in its SSU rRNA gene than any other published Acrasidae SSU; there is one at each known Acrasidae insertion site (S516, S895, S956, S1199), as well as a novel insertion site (S1211) not previously observed in Acrasidae. Two of the introns, S516 and S956, have an embedded open reading frame (ORF) encoding a putative 189aa His-Cys box homing endonuclease (HEG). The S516 intron's HEG is in the forward direction in frame +2 at 14 bp 3′ of the intron's insertion site. The S956 intron's HEG is in the reverse direction in frame −2 starting at 220 bp from the 3′ end of the 1081 bp intron (Figure 2). These two predicted proteins are not easily aligned with one another and share limited homology, with only a few short stretches (ca. 40 aa) of 30%–40% amino acid identity. The S516 HEG of NJ13 shares 60% amino acid identity to that of the S516 HEG in *Ac. rosea* 1Ba5-1 (GenBank AER08052). The S956 HEG protein of NJ13 blasts (BlastP) to a HEG protein found on a short contig of the genome of *Ac. kona* strain ATCC MYA-3509 (JAOPGA020000628.1, Sheikh et al. 2024). This 2114 bp *Ac. kona* genomic contig blasts (BlastN) to the SSU rRNA gene (HM114344) of the same strain but only shares a short 72 bp homologous stretch with just 81% nucleotide sequence identity. The contig itself does not appear to be a rRNA gene, only having this short stretch of the SSU and the coding sequence for a HEG. Introns S516 of *P. rosea* HI12 and NJ13 are nearly the same length (1026–1027 bp) and the last 874 bp of the introns are nearly identical. However, the first 152 bp, including where the start codon of a functional HEG would be (if present), are dissimilar and not alignable. Contrary to strain NJ13, no in-frame start codon is present in strain HI12, and all conceptual translations lead to frameshifts and no obvious HEG ORFs. Future investigation of introns and HEGs is necessary to tell the full story of intron evolution within Acrasidae.

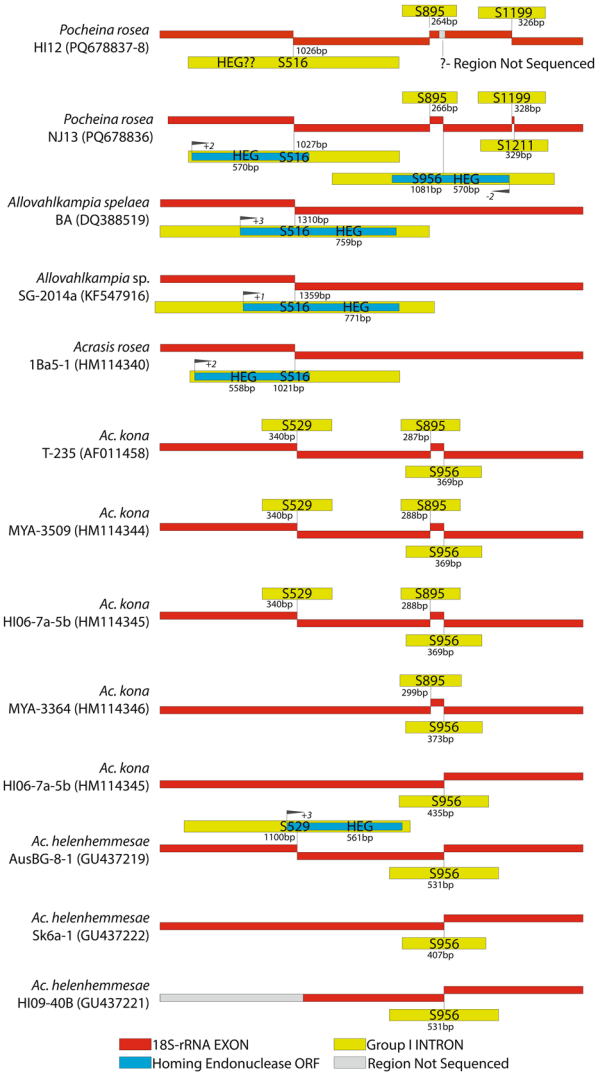

FIGURE 2 | Map of known group I introns and homing endonucleases in Acrasidae nuclear-encoded SSU rRNA genes. The location of each intron is depicted with SNNN, representing the homologous site in the 16S rRNA gene of *E. coli*. Red lines are rRNA coding regions. Yellow lines are group I introns. Blue boxes within group I introns are homing endonuclease open reading frames (ORF). Gray lines are regions that were not sequenced. All lines are to scale.

3.2.2 | Molecular Phylogeny

The topology of the SSU phylogeny (Figure 3) shows that all newly isolated *Pocheina* spp. form a fully supported clade with 100% Maximum Likelihood bootstrap support (BS) and Bayesian posterior probability (PP) of 1.0 within a fully supported (100%/1.0) Acrasidae *sensu* Brown, Silberman, et al. (2012). Both *P. rosea* isolates (NJ13 and HI12) branch together with high support (100%/1.0) and are sister to the single *P. flagellata* (HUNT2) SSU sequence. However, the sequence identified as "*P. rosea*" LOST07L112 in Brown, Silberman, et al. (2012) remains problematic because it branches within the genus *Acrasis* in a fully supported clade of the species *Ac. rosea*. We will later demonstrate that this sequence is a contaminant from a verified *Ac. rosea* isolate, and as such, the genus *Acrasis* is recovered as a highly supported monophyletic group in SSU trees (98%/1.0) and now completely conforms to the morphological-based species concept of Brown, Silberman, et al. (2012), Dykstra (1977), and Page and Blanton (1985). A clade with moderate BS and full PP support containing soil amoeba AND12 (AY965862) and all *Allovahlkampfia* spp. is recovered (71%/1.0). This is congruent with the results of Geisen et al. (2015), although they recovered higher ML support for the clade (possibly because of differing taxa in their analyses). There is limited resolution among the major Acrasidae lineages, but the SSU tree shows a basal split between *Acrasis* and a poorly supported group comprising *Pocheina* as sister to *Allovahlkampfia* spp. (65%/0.92). Overall, three monophyletic lineages are resolved in SSU analyses of Acrasidae: *Acrasis*, *Pocheina*, and *Allovahlkampfia*.

Although the short and highly conserved 5.8S gene has limited resolving power, most of the salient interpretations inferred from the SSU phylogenetic analyses are recovered in 5.8S trees (Figure 4). In the 5.8S analyses, Acrasidae is recovered as a fully supported clade (100%/1.0), though the genus *Acrasis* appears paraphyletic. Consistent with the SSU phylogeny, each species of *Acrasis* is recovered with strong support. Most notable is that with greater taxon sampling, the genus *Pocheina*, including LOST07L112, is fully supported (100%/1.0) (and weakly sister to *Allovahlkampfia*).

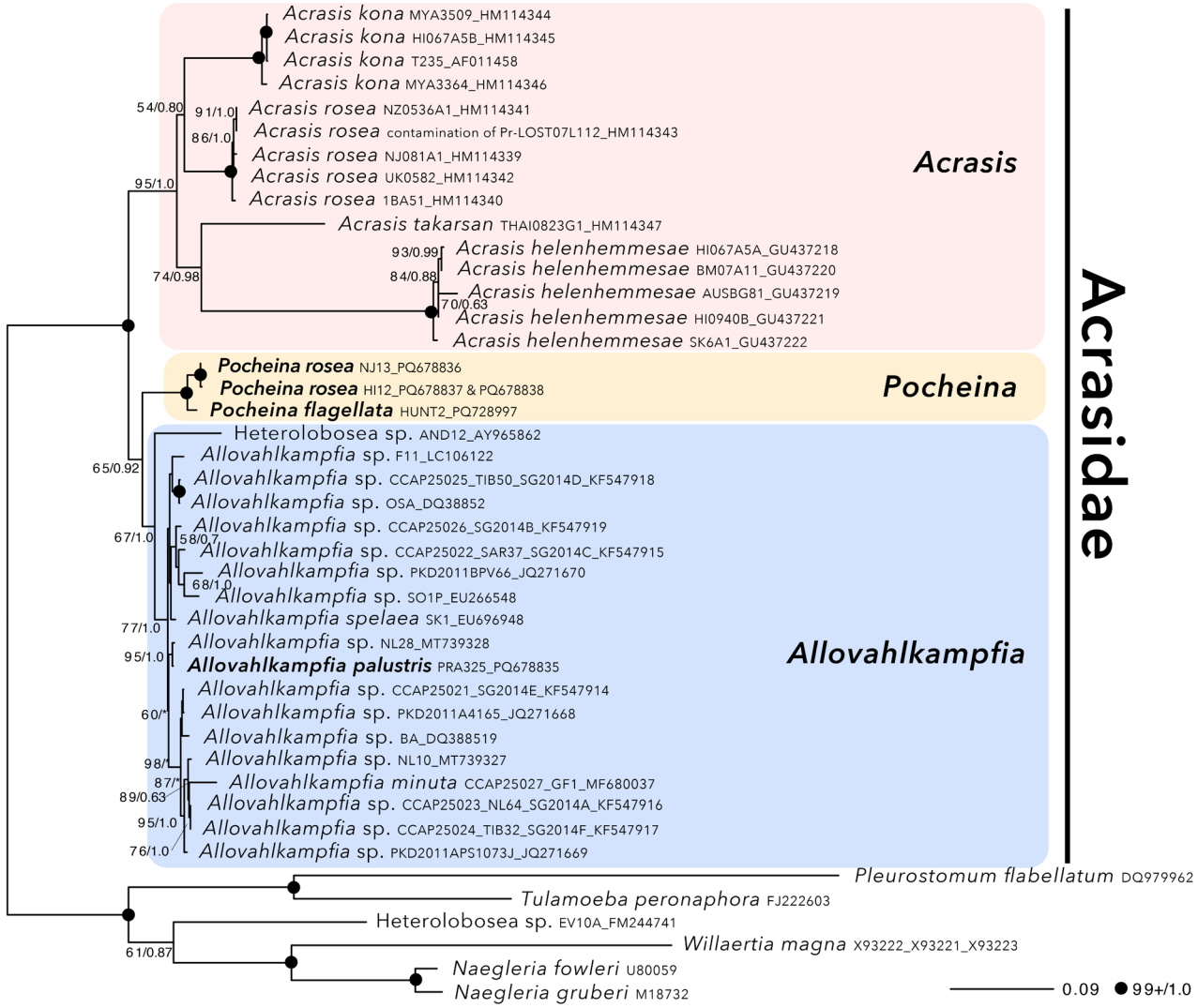

FIGURE 3 | Maximum likelihood SSU rRNA gene tree of Acrasidae with closely related heteroloboseans as an outgroup, using RAxML with GTR + G model of substitution. Bootstrap support values and Bayesian posterior probabilities (see Section 2) are shown at nodes with support values above 50%/0.7, respectively. An asterisk (*) denotes PP below 0.5. Our novel data are bolded.

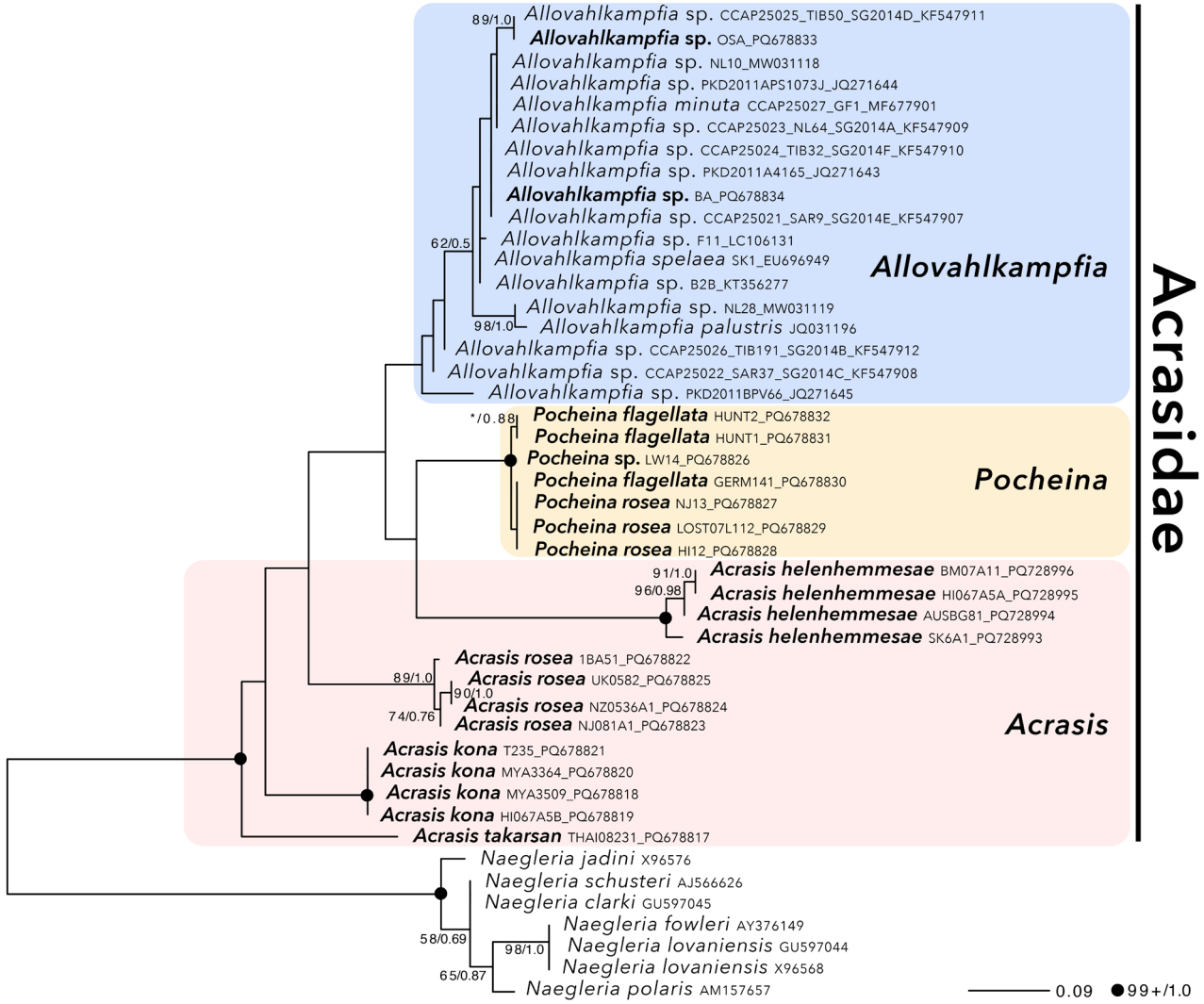

FIGURE 4 | Maximum likelihood 5.8S rRNA gene tree of Acrasidae with closely related heteroloboseans as an outgroup, using RAxML with the GTR + G model of substitution. Bootstrap support values and Bayesian posterior probabilities (see Section 2) are shown at nodes where both values are above 50% or 0.5, respectively. The asterisk (*) denotes BS below 50%. Our novel data are bolded.

The strongly supported and conflicting position of "*P. rosea*" LOST07L112 sequences in their respective SSU and 5.8S rRNA gene phylogenetic trees is problematic (Figures 3 and 4). The SSU sequence is fully supported as a member of *Ac. rosea*, while the 5.8S sequence is fully supported as a member of the genus *Pocheina*. Fortunately, analyses of the independently generated SSU and ITS regions from all *Pocheina* and *Acrasis* isolates provide a logical resolution to this phylogenetic inconsistency.

3.2.3 | Discordant Phylogenetic Signal Due to Contamination

When the "*P. rosea*" LOST07L112 SSU sequence was generated (Brown, Silberman, et al. 2012), there were no other molecular data from other *Pocheina* strains. Sequence comparisons became possible only when we generated molecular data from new *Pocheina* isolates, particularly the ITS region from each of our *Pocheina* spp. and *Acrasis* spp. strains, which overlap to varying extents with the independently amplified SSU rRNA genes reported in Brown, Silberman, et al. (2012). The first piece of hard evidence that the "*P. rosea*" LOST07L112 ITS region and SSU (HM114343) sequences do not originate from the same organism is that there are 18 nucleotide differences among the overlapping 105 bp SSU gene(s) shared between the separately amplified ITS and SSU regions (Figure 5), even though the exact same genomic DNA was used to assemble the amplification reactions. This contrasts with the minimal intra-strain sequence differences of this region among all *Ac. rosea* isolates generated by SSU amplifications (0–2 bp), and the 100% SSU sequence identity among all the *Pocheina* spp. ITS amplicon and transcriptome generated sequences. The pairwise sequence difference between the SSU and the ITS amplicons of "*P. rosea*" LOST07L112 is well outside this range.

We then determined which gene sequence belongs to *Pocheina* and which to the contaminant. Because all our newly generated ITS sequences are contiguous with the SSU rRNA gene, we were able to link each ITS to its corresponding SSU rRNA

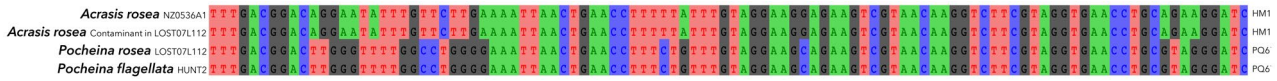

FIGURE 5 | Image of an alignment of the 3′ end of the SSU from LOST07L112 from Brown, Silberman, et al. (2012) (HM114343) and from the ITS region amplicon obtained in this study. The top line is the sequence from *Acrasis rosea* NS05-36a-1 (HM114341). The bottom line is from *Pocheina flagellata* HUNT2 obtained in this study.

gene. This was not possible with SSUs amplified in the Brown, Silberman, et al. (2012) study because the 3′ reverse PCR primers were within the SSU gene. The nearly complete SSU and ITS sequences of *P. flagellata* (HUNT2)/*P. rosea* (NJ13, HI12) can each be assembled into a contiguous contig with 100% sequence identity in the overlapping SSU gene, and the SSU and 5.8S phylogenetic tree topologies are congruent. The same is true for all *Acrasis* spp. isolates except for "*P. rosea*" LOST07L112. Besides sorocarp morphology and the 100% SSU sequence identity among all contiguous *Pocheina* ITS region sequences (discussed above), the remainder of the LOST07L112 ITS can be fully aligned with those from all other *Pocheina* isolates and lacks similarity to the ITS region of any *Acrasis* isolate. Thus, multiple lines of evidence indicate that the ITS region sequence of "*P. rosea*" LOST07L112 originated from *Pocheina*.

On the other hand, we can confidently assign the SSU sequence attributed to '*P. rosea*' LOST07L112 (HM114343) to an *Ac. rosea* contamination. There is no branch length between HM114343 and *Ac. rosea* NZ0536A1 (HM114341) in SSU phylogenetic analyses (Figure 3) and close examination of their edited SSU sequences reveals that they are 100% compatible with one another. These sequences differ in only eight positions, where mixed peaks on the sequencing chromatograms of one were fully resolved to a compatible base in the other (Table 4 of Brown, Silberman, et al. 2012). The amplicon yielding HM114343 likely originated from a pipetting error of *Ac. rosea* NZ0536A1 DNA into the LOST07L112 PCR tube during the assembly of the SSU amplification reaction. This is very plausible because the PCRs from Brown, Silberman, et al. (2012) were set up in concurrent experiments. Thus, we are reassigning the "*P. rosea*" LOST07L112 SSU sequence to *Ac. rosea* LOST07L112 (HM114343).

3.3 | Systematics of Acrasidae

Based on fruiting body morphology, members of the genus *Acrasis* and of the genus *Pocheina* are readily distinguishable on the primary isolation substrates, which thus far comprise plant materials such as bark, leaves, or inflorescences (Brown et al. 2010; Brown, Silberman, et al. 2012; Olive et al. 1983). Members of *Allovahlkampfia* are currently circumscribed by rRNA sequence data, and the clade comprising the genus is only moderately supported in single gene phylogenies (Figures 3 and 4) although there is increased support in concatenated SSU + 5.8S trees (Figure 6; Gao et al. 2022). Even though there are some morphological differences among the three described species of *Allovahlkampfia* (Anderson et al. 2011; De Obeso-Fernández del Valle and Maciver 2017; Walochnik and Mulec 2009), it is unclear if they are taxonomically informative. However, *Allovahlkampia* was annotated into five groups based on unrooted phylogenetic analyses of the entire ITS region (Gao et al. 2022). The newly sequenced ITS region of *Allovahlkampfia* strains BA and OSA branches with Group 2 and Group 3, respectively (Figure S1). Molecular phylogenetic analyses containing more genes should better delineate *Allovahlkampfia*, which may lead to the recognition of taxonomically informative characteristics, as was the case with *Acrasis* (Brown, Silberman, et al. 2012).

The newly sequenced ITS region of all *Pocheina* isolates, the *Acrasis* isolates from Brown, Silberman, et al. (2012) and *Allovahlkampfia* isolates OSA and BA, was used to test the taxonomic concepts advocating that genera are monophyletic in 5.8S phylogenetic trees and that species can be delineated by unique ITS1/2 sequences (De Jonckheere 2004; De Jonckheere and Brown 2005). The fact that genus *Acrasis* is well supported and is comprised of species that are readily identifiable using a combination of morphological and SSU molecular data (Brown, Silberman, et al. 2012) provides an independent assessment of the rigor of these molecular data for taxonomic assignments.

The 5.8S rRNA gene delineation of genera is not especially useful in Acrasidae because of its limited resolution in conjunction with the long branch leading to outgroup taxa; this shortcoming is apparent in the inferred paraphyly of the genus *Acrasis* and the weakly supported clade of *Allovahlkampfia* (Figure 4). However, 5.8S phylogeny does recover the genus *Pocheina* with full support (Figure 4). Concatenation of 5.8S and SSU sequences and even SSU alone have enough information to robustly delineate genera among the Acrasidae (Figures 3 and 5).

We have multiple isolates of 'good' species within *Acrasis* to assess whether each species possesses unique ITS sequences. Considering only ITS sequences (not including the 5.8S), there are intra-specific sequence differences in most species for which we have more than one isolate (Table S1). Except for *Ac. kona* strains (which have identical ITS1 and ITS2), no *Acrasis* species has a completely unique ITS shared exclusively among strains. The most extreme example of intra-specific ITS sequence diversity is from the morphologically simplest *Acrasis*, *Ac. helenhemmesae* (Brown et al. 2010). This species has the longest ITS of all currently known *Acrasis* species (Table S1). Among the four *Ac. helenhemmesae* isolates, the ITS regions (ITS1 and ITS2, excluding the 5.8S rRNA coding gene) range from 3094 to 4275 nucleotides in length. Not only are there length differences, but scattered between regions of sequence similarity, there are multiple regions that are unalignable. Much of this can be attributed to numerous direct and inverted repeats within the ITS, which also account for some of the intra-specific ITS sequence length differences (Figure S3). Thus, the hypothesis of species delineation based on unique ITS sequences (De Jonckheere 2004; De Jonckheere and Brown 2005) does not hold for *Acrasis and* may not be applicable for *Pocheina*. The only *Pocheina* strains

FIGURE 6 | Maximum likelihood tree of concatenated Acrasidae SSU and 5.8S genes, shown rooted as in Figure 3, using RAxML with GTR + G model of substitution. Bootstrap support values and Bayesian posterior probabilities (see Section 2) are shown at nodes where both values are above 50% or 0.5, respectively. An asterisk (*) denotes BS or PP below 50% or 0.5, respectively. Taxa in which a gene is missing are denoted as "no SSU" or "no 5.8S." Taxa for which novel data are presented here are bolded.

sharing identical ITS sequences are HUNT 1 and 2 (which are likely the same strain; see below). Until more morphological and genomic data are available for members of *Allovahlkampfia* and *Pocheina*, it is premature to utilize ITS region data for genus and species designations. We can only urge caution when establishing molecular barcodes in the absence of independently verifiable taxonomic criteria.

Incorporating molecular data into a species concept for members of the genus *Pocheina* is in its infancy. Currently, there is a paucity of isolates to study, and it seems to be relatively rare in the environment, as it is rarely observed or reported. We simply have too few isolates to confidently determine the taxonomic significance or stability of morphological variations observed in any life-history stage (this study; Olive et al. 1983), especially as our isolates could not be cultured, and thus are not amenable to growth in a "common garden" environment. Replicates are required to assess the support and stability of an observation. Our only example comes from *P. flagellata* HUNT 1 and 2, which were recovered from the same tree, 5 years apart. Germinating trophozoites possessed the same morphologies and are nearly identical at the molecular level. We interpret this to mean that the tree was colonized by this single strain, which possesses morphological characteristics that are stable over time. Additional replicates of other isolates are warranted to test this supposition.

Taxonomic designations are hypotheses that are subject to reinterpretation when additional data become available. We do not have enough information yet to challenge the taxonomic definitions of *P. flagellata* versus *P. rosea* (Olive et al. 1983). However, detailed analyses of the ITS region suggest that their taxonomy may be subject to revisions. The 5.8S phylogenetic tree shows *P. flagellata* GERM branching with the *P. rosea* isolates rather than the other *P. flagellata* isolates (HUNT), albeit with very poor support (Figure 4). Close inspection of the ITS region alignment hints at 'signature sequences' shared between *P. flagellata* GERM and *P. rosea* to the exclusion of *P. flagellata* HUNT (Figure S2). It may turn out that the differing morphology

among flagellates becomes a component in splitting *P. flagellata* into different species. Unfortunately, Olive et al. (1983) illustrate morphologically unique flagellates from multiple strains in their image plates, but the image of the type strain (NC81-87) is not denoted. Thus, interpretation of the morphological characteristics of the type strain of *P. flagellata* Olive et al. (1983) is not possible. More isolates, more data, and additional analyses are needed prior to any taxonomic revision within *Pocheina*. Unfortunately, our attempts to generate a stable culture of *Pocheina* have failed. Without such, generating conclusive morphometric data are not possible or practical.

3.4 | Ancestral Traits of Acrasidae

To date, nearly all *Allovahlkampfia* species have been isolated from soil environments and typically cultivated as amoebae in liquid media, with occasional cultivation on agar plates (Anderson et al. 2011; De Obeso-Fernández del Valle and Maciver 2017; Gao et al. 2022; Geisen et al. 2015). None have been isolated as a fruiting amoeba, unlike *Acrasis* and *Pocheina*, which have exclusively been isolated from fruiting bodies on plant materials. To date, the only *Allovahlkampfia* to be isolated from plant material is strain BA. It was originally isolated from tree bark as an amoeba and propagated in this form. However, a single sorocarp was induced in a study by Brown, Silberman, et al. (2012) that displayed a morphology distinct from both *Acrasis* and *Pocheina*, notably lacking the raised hila on spores. Induction of the sorocarp was achieved by adding amoebae to *Pinus* sp. bark soaked in a water/yeast slurry (Brown, Silberman, et al. 2012). In that same study, *Allovahlkampfia* strain OSA failed to undergo fruiting in similar attempts (Brown, Silberman, et al. 2012).

We are unaware of efforts to induce fruiting body formation in other *Allovahlkampfia* strains. Thus, it is plausible that additional *Allovahlkampfia* are sorocarpic amoebae and that appropriate conditions for inducing cell aggregation and/or fruiting body formation have yet to be discovered. Based on our phylogenetic analyses, it is most parsimonious to propose that social multicellularity and fruiting body formation are ancestral traits in the Acrasidae lineage, as fruiting is observed across major clades. It is possible that some isolates, strains, or species have lost the ability to form fruiting bodies, or that this ability has simply not been observed under laboratory conditions. Therefore, the absence of fruiting should not be considered a taxonomically significant feature.

It is conceivable that *Allovahlkampfia* and *Pocheina* may eventually conform to a morphological and molecular species concept similar to that used for *Acrasis* (Brown, Silberman, et al. 2012). Future efforts to induce cell aggregation and fruiting body formation would be valuable for comparative morphological studies, taxonomic assignments based on multiple independent traits, and gene expression analyses that could elucidate the similarities and differences in cellular aggregation (Sheikh et al. 2024). In a similar vein, given the presence of flagellated cells in *Pocheina* and one undocumented observation in *Acrasis* (see Brown, Silberman, et al. 2012; pp. 104), testing for flagellates throughout the lineage may uncover their occurrence where they are currently undocumented.

4 | Conclusion

Sorocarpic amoebae occur across the eukaryotic tree and are found in all major lineages containing amoeboid taxa. The ability to form fruiting bodies mediated by cell–cell aggregation likely evolved independently at least eight times (Tice and Brown 2022). Most of these lineages are mono-typic genera or, at best, contain just a few species. Examples include *Copromyxa protea* (Tubulinea, Amoebozoa), *Fonticula alba* (Holomycota, Obazoa), *Guttulinopsis* spp. (Cercozoa, Rhizaria), and *Sorodiplophrys stercorea* (Labyrinthulomycetes, Stramenopiles) (Brown et al. 2009, 2010; Brown, Kolisko, et al. 2012; Brown and Silberman 2013; Raper et al. 1977; Schuler et al. 2018; Tice et al. 2016; Tice and Brown 2022). However, sorocarpic amoebae are far from obscure and are charismatic protists. The most famous example may be *Dicyostelium discoideum* (Evosea, Amoebozoa); it is a model organism for the study of cell motility, chemotaxis, pattern formation, host–pathogen interactions, and numerous biomedical processes (Bozzaro 2019; Martin-González et al. 2021). The dictyostelids are speciose and highly successful in soil environments (Sheikh et al. 2018). So far, Acrasidae is the only other group containing a rich diversity of sorocarpic amoebae, comprising three genera and multiple sorocarpic species. Complementing ever-improving traditional culturing and molecular methods for detecting biodiversity, expanded global sampling into underexplored environments (e.g., dead, decaying, or living plant material) is likely to uncover additional acrasid species and perhaps even genera. It is likely that such studies may also reveal additional diversity in other lineages of sorocarpic amoeba, which would provide a wealth of taxa amenable to comparative studies and perhaps even model organism development.

Acknowledgments

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Data Availability Statement

The SSU gene (PQ678835–PQ678838 and PQ728997) and ITS (PQ678817–PQ678834 and PQ728993–PQ728996) sequences are deposited at NCBI. The raw Illumina sequencing transcriptome reads from *P. flagellata* HUNT2 are deposited under the Bioproject PRJNA1192706 on NCBI. The transcriptome assembly from HUNT2 and untrimmed and trimmed SSU and ITS alignments are available on figshare, https://doi.org/10.6084/m9.figshare.27174660.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.