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Limited genetic diversity among *Sarcocystis neurona* strains infecting southern sea otters precludes distinction between marine and terrestrial isolates

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Abstract

Sarcocystis neurona is an apicomplexan parasite identified as a cause of fatal neurological disease in the threatened southern sea otter (*Enhydra lutris nereis*). In an effort to characterize virulent *S. neurona* strains circulating in the marine ecosystem, this study developed a range of markers relevant for molecular genotyping. Highly conserved sequences within the 18S ribosomal gene array, the plastid-encoded RNA polymerase (RPOb) and the cytochrome c oxidase subunit 1 mitochondrial gene (CO1) were assessed for their ability to distinguish isolates at the genus and species level. For within-species comparisons, five surface antigens (SnSAG1-SnSAG5) and one high resolution microsatellite marker (Sn9) were developed as genotyping markers to evaluate intra-strain diversity. Molecular analysis at multiple loci revealed insufficient genetic diversity to distinguish terrestrial isolates from strains infecting marine mammals. Furthermore, SnSAG specific primers applied against DNA from the closely related species, *Sarcocystis falcatula*, lead to the discovery of highly similar orthologs to SnSAG2, 3, and 4, calling into question the specificity of diagnostic tests based on these antigens. The results of this study suggest a

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Conflicts of Interest

The authors have no known conflicts of interest.

population genetic structure for *S. neurona* similar to that reported for the related parasite, *Toxoplasma gondii*, dominated by a limited number of successful genotypes.

Index Keywords

Sarcocystis neurona; sea otter (*Enhydra lutris*); SnSAG; genotyping; population genetics

1. Introduction

Protozoal encephalitis is a major cause of mortality in southern sea otters, a federally listed threatened species (Miller et al., In Press; Thomas et al., 2007). *Sarcocystis neurona*, the agent responsible for deadly neurologic disease in horses (Dubey et al., 2001b), has been shown to cause fatal disease in sea otters and other marine mammals such as Pacific harbor seals (Dubey et al., 2003b; Kreuder et al., 2003; Lapointe et al., 1998; Lindsay et al., 2000; Lindsay et al., 2001; Miller et al., In Press; Miller et al., 2001a; Miller et al., 2004; Miller et al., 2001b; Mylniczenko et al., 2008; Peat, 2005; Thomas et al., 2007). Despite the importance of *S. neurona*, current research efforts addressing neurologic disease in sea otters have focused primarily on the zoonotic pathogen, *Toxoplasma gondii. Toxoplasma gondii* is a closely related tissue cyst-forming coccidian that shares many life history traits in common with *S. neurona.* It is likely that mechanisms resulting in the establishment of these terrestrial pathogens in the marine environment are very similar and may have selected for unique, marine-adapted strains. Studies on *T. gondii* for instance, discovered a novel genotype X associated with fatal disease in sea otters (Miller et al., 2004). Evidence for the presence of Type X strains was also found in intermediate and definitive hosts inhabiting the adjacent terrestrial environment as well as a marine dwelling, filter-feeding invertebrate, which is a major staple in the sea otter diet (Miller et al., 2008). These findings, combined with laboratory studies confirming the ability of filter-feeding invertebrates to concentrate and harbor infectious *T. gondii* oocysts (Arkush et al., 2003; Lindsay et al., 2004), suggest a mechanism of land-to-sea flow involving oocysts shed by definitive felid hosts being washed to sea via freshwater runoff, with subsequent bio-concentration in tissues of filter-feeding invertebrates consumed by sea otters. Whether the same is occurring for *S. neurona* has not been examined because high resolution, DNA sequence-level molecular tools to discriminate among *S. neurona* isolates are lacking.

In an effort to better characterize strains of *S. neurona* circulating in the marine environment, and to establish meaningful genotyping methods, this study developed genetic markers with potential for identifying and distinguishing parasites at the genus, species and sub-species level. We then applied these markers to determine and quantify the genetic variability of *S. neurona* isolated from southern sea otters in comparison with strains isolated from other marine and terrestrial mammal hosts.

2. Materials and methods

2.1 Parasite DNA

Sarcocystis neurona DNA samples were obtained from infected tissues collected at necropsy or from isolates obtained from infected tissues using cell culture, resulting in a total of 25 marine and 5 terrestrial mammal samples. The marine mammals included 22 sea otters (*Enhydra lutris nereis*) (Miller et al., In Press; Peat, 2005) and three harbor seals (*Phocina vitulina*) (Miller et al., 2001b), all of which died with systemic protozoal infections and were found stranded along the Pacific coast of California, USA (see Table 1 for details). Terrestrial isolates included three from California horses (*Equus caballus*) (Marsh et al., 1996; Marsh et al., 1999; Peat, 2005) diagnosed with equine protozoal myeloencephalitis

(EPM) and two strains from presumably asymptomatic raccoons (*Procyon lotor*) isolated in Wisconsin, USA (Sundar et al., 2008). To test for specificity, primer sets were also tested on DNA from the related parasites *Sarcocystis falcatula*, *Sarcocystis cruzi*, *Sarcocystis campestris*, *Toxoplasma gondii*, and *Neospora caninum*.

2.2 PCR amplification and sequencing

Intra-specific variability in SnSAGs has been reported previously (Crowdus et al., 2008; Howe et al., 2008), so we decided to exploit this property and apply these markers against the *S. neurona* infected samples to resolve strain genotype differences. To maximize the likelihood of detecting strain-specific polymorphisms at these loci, primer sets were designed to amplify the majority of the open reading frame from all known SnSAG loci (SnSAG1-5). SnSAG1 and SnSAG5 are encoded by two different, paralogous genes. Strains of *S. neurona* possess one or the other gene (Crowdus et al., 2008). Primers (designated SnSAG1-5-6) were developed within conserved sequences of the two genes in order to amplify both of these mutually exclusive surface antigens. To differentiate isolates that encoded SnSAG1 from those that encoded SnSAG5, SnSAG1-specific primers were developed. Primers were designed using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000) based on the following GenBank sequences: SnSAG1 (AY032845), SnSAG2 (AY191006), SnSAG3 (AY191007), SnSAG4 (AY191008), SnSAG1-5-6 (AY170620). Primer sequences are provided in Table 2.

Previously published primer sets used include those for the cytochrome c oxidase subunit 1 (CO1) mitochondrial gene (Inagaki et al., 1997), internal transcribed spacer region 1 (ITS1) of the nuclear ribosomal gene array (Miller, RH, Grigg ME et al., unpublished), the apicoplast-encoded RNA polymerase (RPOb) (Dubey et al., 2003a), and microsatellite marker Sn9 (Asmundsson and Rosenthal, 2006). Highly conserved, multi-copy loci, such as CO1, ITS-1, and RPOb were utilized to identify and distinguish *S. neurona* strains infecting marine mammals from infections by related Apicomplexans at the genus and species level (Dubey et al., 2003a; Miller et al., submitted; Peat, 2005). Mitochondrial CO1 and apicoplast RPOb are extranuclear loci relevant phylogenetically as a marker to trace maternal inheritance and document sexual events in *S. neurona* life history. RPOb also has been used previously to distinguish *Sarcocystis* at the genus and species level (Dubey et al., 2003a). Sn9 is a polymorphic microsatellite marker that has been used successfully for intraspecific analysis of *S. neurona* (Asmundsson et al., 2006; Sundar et al., 2008). To increase the sensitivity of previously published RPOb and Sn9 primer sets, a set of forward and reverse primers external to those published by Dubey et al. (2003a) for RPOb were developed, and Sn9 primers were made into a hemi-nested set by the addition of an external forward primer to those developed by Asmundsson and Rosenthal (2006) (see Table 2 for sequences).

PCR was conducted on 1.5μl of each DNA extraction sample with 5 μl of PCR Buffer (10X containing $MgCl_2$, Sigma), 5 μl of 2 mM dNTP mix (Fermentas), 50 pmol of each primer and 1.25–2.5U of Taq Polymerase (Sigma), with the total reaction volume reaching 50 μl. PCR amplification was conducted for 35 cycles and consisted of 94°C for 5 minutes, 94°C for 40 seconds, 58°C for 40 seconds, 72°C for 90 seconds and 72°C for 10 minutes. PCR amplification products were visualized on 0.8% agarose gels stained with GELRED dye (Biotium). Negative controls for the external and internal reactions consisted of molecular grade de-ionized water. All PCR products were purified using ExoSAP-IT (USB) according to the manufacturer's instructions and sequence confirmed with forward and/or reverse reads by Rocky Mountain Laboratory Genomics Unit DNA Sequencing Center, Division of Intramural Research, Hamilton, Montana.

2.3 Sequence analyses

Sequences were visualized using FinchTV software and analyzed using the Seqman application of the Lasergene software suite. Nucleotide and protein sequence alignments were created with ClustalW2 (Larkin et al., 2007). Closest *T. gondii* and *N. caninum* orthologs to the SnSAGs were assessed using BLASTp function available on ToxoDB (Gajria et al., 2008).

3. Results

Molecular tool development and isolate characterization

The specificity of each primer set was evaluated by testing against DNA from related parasites. At ITS-1 and RPOb, all coccidia tested amplified for both markers except RPOb in *S. cruzi* (Figure S1A) (RPOb data not shown). SnSAG1 and SnSAG1-5-6 primers were shown to be *S. neurona* specific. In contrast, SnSAG2, SnSAG3, SnSAG4, and Sn9 amplified both *S. neurona* and *S. falcatula* DNA (Figure S1A). DNA sequencing of the PCR products confirmed *S. neurona* was present in all specimens collected and enabled molecular characterization of *S. neurona* strains. Representative genomic DNA sequences for each *S. neurona* allele were deposited in GenBank (SnSAG1: GQ851951; SnSAG2: GQ851952; SnSAG3: GQ851954 and GQ851955; SnSAG4: GQ851957 and GQ851958; SnSAG5: GQ851960; RPOb: GQ851961; Sn9: GQ865624, GQ865625 and GQ865626). Results for each marker are discussed individually below.

3.1 Internal transcribed spacer region-1 (ITS-1) of the nuclear ribosomal gene array

The ITS-1 region was amplified with pan-tissue cyst coccidian-specific primers (Miller et al., In Press). Size polymorphisms within the ITS-1 region distinguish *S. neurona* and *S. falcatula* from other tissue cyst-forming coccidia known to infect sea otters and other animals investigated in this report (Figure S1A). DNA sequencing of the PCR products identified only *S. neurona*; no animals were found to be infected with *S. falcatula* (data not shown).

3.2 Mitochondrial cycloxygenase 1 (CO1)

Resolution of the CO1 sequence was limited to samples from which live *S. neurona* parasites were isolated in cell culture, primarily because contaminating host DNA from the infected tissues will also amplify using these primers. All *S. neurona* isolates that did amplify gave one unambiguous CO1 sequence that did not differ from previously reported sequences (Peat, 2005) (data not shown).

3.3 Apicoplast RNA polymerase beta-subunit (RPOb)

RPOb primers amplified ~700 bp product from all coccidia tested except *S. cruzi*. All *S. neurona* RPOb sequences in this study were identical and differed at four polymorphic sites from the only other *S. neurona* RPOb sequence published, which was a South American isolate (Dubey et al., 2001a). Interestingly, the sequences from this study possessed only a single nucleotide polymorphism from a sea otter isolate sequence originally described as an unidentified *Sarcocystis sp.* (Dubey et al., 2003a). The North American *S. falcatula* sequence identified in this study (GenBank Acc. Number GQ851962) also showed polymorphisms compared to previously published South American *S. falcatula* isolates(Dubey et al., 2003a). The *T. gondii* RPOb gene was amplified from SO4167 tissues and this sequence did not differ from those previously published (data not shown). This confirmed that SO4167 was dually infected with both *S. neurona* and *T. gondii*. When applied against *Sarcocystis campestris* DNA, the RPOb primers yielded a single sequence

that was 95% homologous to the *S. neurona* sequences and represents the first sequence reported for this species (GenBank Acc. Number GQ851963).

3.4 SnSAG genes

3.4.1 SnSAG2, SnSAG3, SnSAG4—The *S. neurona* isolates in this study were PCR and sequence positive for SnSAG2, SnSAG3, and SnSAG4. All SnSAG2 sequences identified in this study were identical. SnSAG3 and SnSAG4 sequences identified two alleles for each of these loci. For SnSAG3, alleles differed at two nucleotide positions and one polymorphism was non-synonymous, resulting in a proline for alanine substitution at amino acid 87. SnSAG4 alleles differed by a single nucleotide polymorphism located within in the intron.

Highly conserved orthologs to SnSAG2, 3 and 4 genes were also identified with the same primer sets in the closely related species, *S. falcatula*. Nucleotide sequences for *S. falcatula* SAG2 and SAG4 shared 95% or greater identity with *S. neurona*. The SAG3 *S. falcatula* sequence possessed a 90 base pair indel in its intron and shared 95% identity with *S. neurona* in the coding region of the gene. GenBank accession numbers for SfSAG2, SfSAG3, and SfSAG4 are GQ851953, GQ851956, and GQ851959, respectively.

3.4.2 SnSAG1-5-6—SnSAG1 primers yielded amplification products from 11 of the 30 SnSAG1-5-6 positive samples. DNA sequencing with both primer sets yielded one allele and confirmed that the 11 SnSAG1 positive samples possessed the SnSAG1 gene. Sequencing of the remaining 20 SnSAG1-5-6 positive PCR amplicons identified two sequences. Eighteen samples possessed an identical sequence identified as SnSAG5. The SnSAG5 allele identified in this study was identical to that reported by Crowdus et al. (2008) but differed from the SnSAG5 gene reported for the isolate SnMU1 (Hyun et al., 2003) by six nucleotide polymorphisms and two indels.

The remaining two samples (SO3523 and SO3639) possessed a unique ~1kb nucleotide sequence that shared 71% and 83% identity with SnSAG1 and SnSAG5 respectively and had a predicted protein sequence of 281 amino acids (GenBank Acc. Number GQ851950). It is presumably a new, previously undescribed surface antigen that we refer to as SnSAG6. Nucleotides 155–700 of this sequence were identical to a partial sequence previously reported from a sea otter *S. neurona* isolate (Crowdus et al., 2008). All samples that were negative at the SnSAG1 locus were positive for either SnSAG5 or SnSAG6, and no isolate was sequence positive at more than one of these loci (Figure S1B).

3.4.3 SnSAG orthologs—To examine whether SAG1-6 genes had sequence correspondence with their counterparts in related apicomplexan parasites, closest-orthologs to SnSAG antigens were identified in *N. caninum* and *T. gondii* using the BLASTp function available on ToxoDB (Gajria et al., 2008). The closest *T. gondii* ortholog to SnSAG1, 3, 4, 5 and 6 was TgSRS28, while the closest hit for SnSAG2 was TgSRS51. *Neospora caninum* orthologs consisted of hypothetical or putative SRS domain containing proteins. These analyses reveal the important point that the similarity of nomenclature of SAG1-6 genes in *S. neurona* is not related directly to sequence similarity to their counterparts in related Apicomplexan parasites. Results are summarized in Supplementary Table S1.

3.5 Sn9 microsatellite

Sn9 showed the greatest number of *S. neurona* alleles (3) among the genotyping markers utilized in this study. Alleles were assigned by direct DNA sequencing and were based on visual inspection of the number of GT repeats detected in the sequence reads. The three alleles identified were of 14, 17, and 18 repeats at frequencies of 6/30, 13/30, and 10/30 isolates respectively (Table 1). The isolate from harbor seal, HS2224, was negative at this

locus. The Sn9 allele amplified from *S. falcatula* (8 repeats) could also be resolved based on a species-specific polymorphism seven nucleotides downstream from the repeat region (GenBank Acc. number GQ865627).

3.6 Isolate diversity

Sequence analysis among the antigen-coding loci (SnSAG1, 3, 4, 5 and 6) allowed for the detection of five distinct multilocus sequence types among the *S. neurona* isolates. The genotypes were assigned based on the presence of SnSAG1, 5, or 6 genes and the inheritance pattern of alleles present at SnSAG3 and 4. With the addition of the single microsatellite marker, the number of genotypes distinguished increased to six (Table 1). The two most abundant genotypes isolated from the sea otters appeared to cluster geographically with genotype II found exclusively in northern locations whereas genotype I was more prevalent along the south-central California coast (Table 1). Also of interest was the finding that the isolates from raccoons (R1 and R2) from Wisconsin had an identical genotype to a harbor seal (HS1423) isolate from California (Table 1).

4. Discussion

The typing scheme developed in this study revealed minimal genetic diversity in marine *S. neurona* isolates at the loci examined. A lack of diversity at the conserved ITS-1, CO1 and RPOb loci among *S. neurona* isolates was expected as the utility of these markers is to confirm infection by *S. neurona*. Size and sequence polymorphisms within the ITS-1 proved useful for discriminating *S. neurona* from other parasites in tissues collected and necropsy. A lack of allelic diversity and cross-reactivity with host DNA hampered the ability of CO1 to characterize strains and predict maternal inheritance patterns. However, this locus did facilitate confirmation of species identity for those strains where tissue culture derived merozoite DNA could be amplified as well as some infected tissue samples. Polymorphisms noted in the RPOb sequence between North American *S. neurona* and *S. falcatula* strains described in this study compared to previously published sequences from South American isolates (Dubey et al., 2003a), may be indicative of sustained geographic isolation. Intra- and interspecific polymorphisms at this locus could prove useful for global studies of phylogenetics and the population genetic structure of *S. neurona* and other *Sarcocystis spp.*, though more South American samples are needed for an accurate assessment. The high similarity of the RPOb sequence from an unidentified *Sarcocystis sp*. (Dubey et al., 2003a) with RPOb sequences recovered from sea otters in this study may suggest that either the sea otter was infected with a closely related *Sarcocystis spp.* or was infected with *S. neurona* harboring a novel RPOb allele, or both. Further genetic characterization is needed to distinguish amongst these scenarios.

This study showed that known SnSAG antigens, SnSAG1-5, as well as SnSAG6, possessed a limited number of alleles among the *S. neurona* strains examined. Only one allele was identified for the SnSAG1, SnSAG2, SnSAG5 and SnSAG6 loci. This lack of allelic diversity within the SnSAGs was unexpected, especially considering that orthologous loci have been shown to be highly informative genotyping markers among clonal strains of the related parasite, *T. gondii* (numerous citations, initial studies include: Grigg et al., 2001; Howe and Sibley, 1995; Parmley et al., 1994; Sibley and Boothroyd, 1992). However, the allele at SnSAG5 did differ from a single, previously published isolate, Sn-MU1 (Hyun et al., 2003), by six nucleotide polymorphisms and two insertion/deletions, all of which resulted in a single, conservative amino acid change. SnSAG3 and SnSAG4 also identified two alleles: two nucleotide polymorphisms resulted in one amino acid change for SnSAG3; and a single nucleotide polymorphism was detected in the SnSAG4 intron. These findings suggest that the diversity previously reported at the SnSAG4 locus, which was based on western blot analysis, was due to cross reactions of polyclonal antibodies to conserved

regions of other proteins (Howe et al., 2008). This study also identified highly conserved (>90% identity for SnSAG2 and SnSAG4, >80% identity for SnSAG3) orthologs to SnSAG2, 3, and 4 in the closely related species *S. falcatula*, a finding that has implications for the specificity of diagnostic tests based on PCR and antibodies for these proteins (Ellison et al., 2003; Hoane et al., 2005).

The minimal diversity of SnSAG2-4 both within and between species might suggest that the function of these proteins is essential to parasite growth and survival and warrants further study. The mutual exclusiveness of SnSAG1, 5, and 6 presents an intriguing phenomenon that has yet to be explained. Whether they represent highly diverged alleles of the same antigen (Elsheikha and Mansfield, 2004) or are the result of a gene duplication event, similar to that described for SAG5B and SAG5C in *T. gondii* (Jung et al., 2004), cannot be determined until more sequence data are available. As research proceeds in discovering the function of these proteins, taking a comparative approach that accounts for loci conserved in related tissue cyst-forming coccidia will likely produce revealing insights into the biology of these important parasites.

The molecular characterization based on differential segregation of SnSAG alleles and a single microsatellite marker did not resolve *S. neurona* strains infecting marine mammals from terrestrial isolates. The overall genetic diversity among *S. neurona* strains examined was surprisingly minimal, and just a few multilocus genotypes were identified. This result could simply reflect the sample set used in this study, since it was composed predominantly of strains isolated from diseased animals, the majority of which were sea otters. It is also possible that certain strains found infecting only sea otters may represent marine-adapted strains, but to resolve this question, additional *S. neurona* isolates from terrestrial animals will need to be studied. Focusing on the opossum definitive host will likely determine the true diversity present in the *S. neurona* population and should allow for specific questions of *S. neurona* biology to be addressed, such as the biological plausibility and potential source(s) of land-to-sea flow of this pathogen. This is especially relevant since the two genotypes identified in infected horses and raccoons in this study were also found in the marine environment.

The application of the robust, highly sensitive, new genetic markers capable of amplifying parasite DNA from infected tissue samples supported previous results (Sundar et al., 2008) indicating that *S. neurona* genotypes clustered geographically along the California coast. Future studies should be focused on whether the apparent geographic dominance of certain strains in sea otters reflects the prevalence of these same strains in the adjacent terrestrial environment. The localized clonal dominance along the California coast, combined with the finding that the same genotype infected raccoons from Wisconsin and a harbor seal from California, raises intriguing questions about the transmission dynamics and life history traits of this organism. Determining a mechanism for how one strain can dominate on a local or global level will provide key insights to epidemiological phenomena of this parasite. Development and application of additional high resolution genetic typing markers should prove a valuable tool for future studies in this regard.

In addition, isolates from asymptomatic hosts will be needed to discern associations between the infecting genotype and disease outcome. The development and utilization of robust typing methods will likely be necessary to allow researchers to ask whether strain “type” is a predictor of severity of disease. A previous study found no association of microsatellite markers and neurologic disease (Asmundsson et al., 2006). However, this and other studies of microsatellite markers (Asmundsson et al., 2006; Sundar et al., 2008) have relied on differences in gel migration to determine size polymorphisms and type isolates; a method subject to individual bias and potential for inconsistency. Also, the study by Asmundsson et

al. (2006) compared disease-causing strains isolated from intermediate/aberrant hosts (4 horses and 1 sea otter) to strains isolated from presumably asymptomatic opossums. It is highly likely that a strain that causes severe disease in an intermediate or aberrant host, such as a sea otter or horse, may cause no disease in the opossum since it is the definitive host. To truly comment on the virulence or disease causing potential of a strain infecting any susceptible host, comparisons should be made between strains isolated from symptomatic and asymptomatic infections of that same susceptible host. The method developed in the current study, that utilized visual counting of sequence reads to determine microsatellite size polymorphisms, presents a new, consistent way to apply these markers that may produce more definitive results. Likewise, a combination of the robust strain-level resolution provided by microsatellite markers and allelic variation at key virulence loci could prove an important tool in this regard, supporting further development of the typing scheme described in this study.

Previous studies have concluded that *S. neurona* possesses an intermediate population structure that exhibits both clonal propagation and recombination (Asmundsson et al., 2006; Elsheikha et al., 2006; Sundar et al., 2008). Analysis of markers in the current study supports these conclusions. The differential segregation of SnSAG alleles indicates past recombination events, and the over-representation of certain strains provides evidence of clonal propagation. This parallels the current understanding of *T. gondii* population structure where sexual recombination provides the driving force for the emergence of successful strains that sweep clonally (Grigg and Sundar, 2009). This intriguing result begs the question whether a single, highly successful strain of this heteroxenous parasite can expand clonally in definitive or intermediate hosts, despite the genetic recombination that occurs during the sexual life cycle stage. Answers to this question are likely, again, to be similar to the closely related species, *T. gondii*, where clonal propagation is explained by 1) its ability to be orally transmitted via carnivorism among intermediate hosts (Su et al., 2003) or 2) by the ability of a single clone to differentiate into both male and female gametes and self-mate in the intestine of the definitive host (Cornelissen and Overdulve, 1985; Pfefferkorn et al., 1977).

5. Conclusion

The multi-locus, DNA sequence level typing methods used in this study revealed spatial clustering superimposed on a background of minimal genetic diversity among *S. neurona* strains infecting sea otters and other marine and terrestrial mammals. The major conclusions of this study are that the conserved markers, ITS-1 and RPOb are useful for genus and species level resolution of tissue cyst coccidia, aiding in the identification of *S. neurona* infection. Examination of allelic segregation at six surface antigen genes (SnSAG1-SnSAG6) and microsatellite marker Sn9 can distinguish genotypes among *S. neurona* isolates, though not at a level of resolution sufficient to discriminate terrestrial isolates from *S. neurona* strains infecting sea otters and other marine mammals. As the microsatellite marker Sn9 identified the greatest number of alleles of the markers analyzed, it is likely that additional microsatellites will further develop this typing scheme to increase resolution. The limited genetic diversity detected in this study, composed of just a few recombinant genotypes, further supports the hypothesis of an intermediate genetic population structure for *S. neurona* in general, characterized by both clonal and sexual propagation. Overall, the evidence for clonality in this heterogamous parasite suggests the potential for self-mating or oral transmission between intermediate hosts, characteristics that were previously thought to be unique to *T. gondii*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Sarcocystis neurona isolate genotypes

			Genetic Marker					
			SnSAG1-5-6	SnSAG3		SnSAG4	SN9	
				239	1056	592	(GT)n	
Sample	**Origin**	**ATOS**		**C**	**C**	**C**		**Genotype**
SO3483	CA	820	5	·	·	·	17	**I**
SO3485	CA	827	5	·	·	·	17	
SO3501	CA	915	5	·	·	·	17	
SO3892	CA	917	5	·	·	·	17	
SO4135	CA	824	5	·	·	·	17	
SO4151	CA	825	5	·	·	·	17	
SO4160	CA	814	5	·	·	·	17	
SO4166	CA	827	5	·	·	·	17	
SO4167	CA	827	5	·	·	·	17	
SO4168	CA	832	5	·	·	·	17	
SO4169	CA	819	5	·	·	·	17	
SO4171	CA	819	5	·	·	·	17	
SO4285	CA	806	5	·	·	·	17	
HS2224	CA	na	1	G	T	·	-	**II**
SO3106	CA	318	1	G	T	·	18	
HS1531	CA	na	1	G	T	·	18	
SO3528	CA	292	1	G	T	·	18	
SO3629	CA	276	1	G	T	·	18	
SO3866	CA	309	1	G	T	·	18	
SO4181	CA	303	1	G	T	·	18	
H1 (SN1)	CA	na	1	G	T	·	18	
H2 (SN3)	CA	na	1	G	T	·	18	
H3 (EPM3)	CA	na	1	G	T	·	18	
HS1423	CA	na	5	·	·	G	14	**III**
R1	WI	na	5	·	·	G	14	

			Genetic Marker					
			SnSAG1-5-6	SnSAG3		SnSAG4	SN9	
				239	1056	592	(GT)n	
Sample	**Origin**	**ATOS**		**C**	**C**	**C**		**Genotype**
R2	WI	na	5	·	·	G	14	
SO3523	CA	141	6	·	·	G	14	**IV**
SO3639	CA	299	6	·	·	G	14	
SO4178	CA	1135	5	·	·	·	18	**V**
SO4194	CA	21	1	·	·	·	14	**VI**

Samples were assigned multi-locus genotypes based on the presence or absence of SnSAG1, 5 or 6 and the differential segregation of alleles for SnSAG3, SnSAG4, and Sn9. Alleles for Sn9 were assigned based on the number of GT repeats resolved by DNA sequencing. Six total genotypes were resolved. Sea otter stranding locations and the resulting isolates are identified by an ATOS (‘as the otter swims’) number which describes the carcass recovery site to the nearest 0.5 km location along the California coast. The numbering system begins north of San Francisco and increases numerically from north to south. Genotype II was found exclusively in or near Monterey Bay and Genotype I dominated to the South in Estero Bay, California. Genotype IV was recovered over a more dispersed area in the Northern half of the sea otter range in central California. SO: sea otter; HS: harbor seal; H: horse; R: raccoon; na: not available.

Table 2

Primers

	Forward Primers	
Marker	**External**	**Internal**
SnSAG1	GGAGGTAAGTGTTGGCGGTA	-
SnSAG2	AGCGGCGTTTTCAGATTGTA	-
SnSAG3	TCAAGGACGTTTTTCCCTGT	CCCTGCCTTTCTGGTCTCTT
SnSAG4	AATACCATACCTCGGCGTCA	-
SnSAG1-5-6	TGCTGCATCATTAGGGTCAG	-
RPOb	TAGTACATTAGAAATCCCTAAAC	† GCGGTCCAAAAAGGGTCAGTGGATATGATWTWTGAAGATGC
CO1	# TYTTGTTYTTYGGICAYCCIGARGTITA	-
Sn9	CTGCTGCTAGCGGACTCTCT	* CGCCAAAAGACTCACAAACA
	Reverse Primers	
	External	Internal
SnSAG1	TCCCGTTTTGGAACAGTAGG	-
SnSAG2	AAAACGAAGGCAAGTGTGCT	-
SnSAG3	CTCTGCATGCTGCAATGAAT	TTCTCCCCAAAGACCATCTG
SnSAG4	TCAAATGGCTGTCTCCACAA	-
SnSAG1-5-6	GCTGTGGGAGTAAGCAGGAT	-
RPOb	TCWGTATAAGGTCCTGTAGTTC	† GCGGTCCCAAAAGGGTCAGTCCTTTATKTCCATRTCT
CO1	# AARTGIGCIACIACRTARTAIGTRTCRTG	-
Sn9	* ACGCGCCTAAACGTGAATAG	-

Primer sets developed in this study using Primer 3 (Rozen and Skaletsky, 2000) are listed from 5′ to 3′. See Materials and Methods for GenBank Acc. numbers of sequences used for primer design. (−): Primer set not developed.

† (Dubey et al., 2003a)

(Inagaki et al., 1997)

* (Asmundsson et al., 2006).