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Molecules infer origins of ectoparasite infrapopulations on tuna

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ABSTRACT

Infrapopulation genetic variation of the oioxenous, hermaphroditic flatworm *Nasicola klawei* (Monogenea: Capsalidae) infecting the nasal cavities of nine yellowfin tuna, *Thunnus albacares*, from the Gulf of Mexico was analyzed using the first internal transcribed spacer (ITS1) single strand conformation polymorphism (SSCP), ITS1 sequencing, and amplified fragment length polymorphism (AFLP). Of a total of 32 worms, six had unique ITS1-SSCP types and the rest was grouped by three types. Two worms of the same infrapopulation shared an ITS1-SSCP type in nine instances but no infrapopulation was monophyletic by ITS1-SSCP analysis. ITS1 sequences (420 bp) varied by 1–11 (0.2–2.6%) nucleotides. Twenty-three AFLP profiles of 80–110 bands failed to support genomic monophyly of any *N. klawei* infrapopulation. 28S rDNA (990 bp) sequences from four worms representing four infrapopulations were identical and matched conspecific GenBank sequences. Concordant ITS1-SSCP and AFLP analyses indicated that these *N. klawei* infrapopulations principally resulted from tuna being repeatedly colonized by planktonic, infective larvae (oncomiracidia) rather than by a single host colonization followed by parasite maturation, self-fertilization, and production of auto-infecting progeny.

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1. Introduction

How hermaphroditic ectoparasites with direct life cycles colonize and proliferate on large, swift-swimming epipelagic marine fishes remains a conundrum. Colonization of the host is a defining action of parasitism and generally regarded as a bottleneck since transmission may oftentimes fail [1]; indeed, such failure seems especially likely for parasites of epipelagic fishes. On the other hand, phylogenetically diverse [2] and species rich [3] ectoparasite communities commonly and predictably exploit particular epipelagic fish species, suggesting that perhaps transmission is routine but that post-infection factors limit infrapopulation size. This parasite-host system is difficult to study because no method exists for observing how and when minute, planktonic parasite larvae colonize and proliferate on seagoing hosts, and large epipelagic fishes are impractical laboratory subjects. As a result, parasitologists lack a basic empirical understanding of how parasites exploit these hosts.

Ectoparasite life history traits like host specificity and mode of transmission can directly affect or be reciprocally informed by quantifying genetic diversity [4]. These immediate, microevolutionary interactions are important because they may ultimately reinforce or lead to macroevolutionary processes such as speciation [5]. Herein, we use molecular techniques to investigate the origins of ectoparasite infrapopulations on epipelagic marine fish. The ectoparasitic flatworm *Nasicola klawei* (Stunkard, 1962) Yamaguti, 1968 (Platyhelminthes: Monogenea: Capsalidae) was chosen for this study because of its curious distribution in nature: we have observed hundreds of infections and all of them comprise an infrapopulation consisting of two adult worms within each nasal cavity of each yellowfin tuna (*Thunnus albacares*) (personal observations, SAB and GWB). This low and predictable number of worms per host facilitated asking a simple question: are the worms infecting each tuna immediate kin or progeny of different parents? And by extension, what is the principal modality of ectoparasite larval transmission in the open ocean: horizontal dispersal or autoinfection?

2. Materials and methods

2.1. Collections

Thirty-two of 36 *N. klawei* observed were collected in July 2009 from the nasal cavities of nine yellowfin tuna (*T. albacares*) captured in the north–central Gulf of Mexico within a 300-km radius from Biloxi, Mississippi, USA (N30°23′42.57″ W88°53′02.54″). All fish examined were infected with *N. klawei* (100% prevalence) (Table 1). Two worms infected each nasal cavity but some nasal cavities were damaged such that our sample comprised four intact worms from each of five yellowfin tuna (Nos. 1, 3, 4, 6, and 7) and three intact worms from each of four yellowfin tuna (Nos. 2, 5, 8, and 9). The posterolateral portion of each worm was preserved in RNAlater

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Table 1
Samples analyzed in the present study.

Fish number	Sample ID[a]	ITS1-SSCP type	ITS1 sequence	28S rDNA
1	1-L1	4		
	1-L2	4		
	1-R1	3		
	1-R2	3		
2	2-L1	3		
	2-R1	4		
	2-R2	1	HQ721182	HQ721184
3	3-L1	4		
	3-L2	9		
	3-R1	9	HQ721177	HQ721185
	3-R2	2		HQ721186
4	4-L1	6	HQ721178	
	4-L2	3		
	4-R1	3		
	4-R2	4		
5	5-L1	4		
	5-L2	5		
	5-R1	9		
6	6-L1	3		
	6-L2	4		
	6-R1	7	HQ721181	HQ721187
	6-R2	3		
7	7-L1	4		
	7-L2	4		
	7-R1	3	HQ721180	GenBank#
	7-R2	3		
8	8-L1	4		
	8-L2	4		
	8-R1	8	HQ721179	
9	9-L1	4		
	9-R1	3		
	9-R2	4	HQ721182	

[a] L, let nasal cavity of tuna; R, right nasal cavity of tuna.

(Qiagen, Valencia, CA) for nucleic acid stabilization and the remainder was vialed in formalin for morphology and identified following Chisholm and Whittington [6]. Vouchers were deposited in the US National Parasite Collection (Beltsville, MD).

2.2. SSCP analysis of ITS1 sequence

DNA was extracted with a DNeasy Tissue kit (Qiagen), quantified using a spectrophotometer NanoDrop (ThermoScientific, Wilmington, DE), diluted to 100 ng/μl, and stored at −20 °C. ITS1 amplification followed Li et al. [7], with primers IT1-forward (5′-GTCGTAACAAGGTTTCCGTAGG-3′) and E5.8S-reverse (5′-GCTGCACTCTTCATCGACGCRCG-3′) amplifying the ITS1 (plus 39–40 bp flanking sequence). SSCP analysis followed Olivares-Fuster et al. [8]. Briefly, 1 μl of restriction product was mixed with 5 μl of denaturing–loading solution (95% formamide, 0.025% bromophenol blue), heated for 5 min at 98 °C and immediately cooled on ice. All 6 μl of denatured products were electrophoresed on GeneGel SSCP non-denaturing polyacrylamide gels (Amersham Biosciences, Piscataway, NJ, USA) rehydrated with SSCP Buffer A pH 9.0 (Amersham Biosciences). A GenePhor electrophoresis unit (Amerham Biosciences) was used with the following running conditions: 5 °C constant temperature, 25 min at 90 V, 6 mA, 5 W plus 60 min at 500 V, 14 mA, 10 W. Gels were silver stained with the DNA Silver Staining Kit (Amersham Biosciences) and digitally photographed.

2.3. DNA sequencing

To confirm ITS1 sequence polymorphisms, the ITS1 of selected individuals was amplified as above. The 5′ terminal variable region (D1–D3) of the 28S rRNA gene was also PCR amplified. Primers used to amplify the 5′ terminal 28S rRNA gene were C1-forward (5′-ACCCGCTGAATT-TAAGCAT-3′) and D2-reverse (5′-TGGTCCGTGTTTCAAGACDNA-3′). PCR products were purified with a High Pure PCR Product Purification Kit (Roche Diagnostic Corp., Indianapolis, IN) and cloned into pGEMTEasy (Promega, Madison, WI). Sequencing reactions were carried out at the Auburn University sequencing core facility using a 3100 Applied Biosystems automatic sequencer (Life Techonologies Co. Carlsbad, CA) with Big Dye terminator sequence chemistry (Life Technologies Co). Two clones from each individual were sequenced to ensure sequence accuracy.

2.4. AFLP analysis

AFLP profiles were determined for twenty-three *N. klawei* individuals (nine worms did not yield high quality DNA and could not be typed) as per Arias et al. [9]. Four specific PCR primer pairs were selected: E.CTT/M.AAC, E.CTA/M.ACC, E.CTT/M.ACC, E.CTA/M.AAC. *Eco*RI primers were labeled at the 5′ end with infrared fluorescent dies IRDye700 or IRDye800 (Li-COR, Lincoln, NE). PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (Li-COR). AFLP profiles were considered unique if <97% similar [9].

2.5. Data analysis

Calculation of similarity values and cluster analysis was carried out with BioNumerics® version 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). Levels of similarity between fingerprints were calculated with the Dice coefficient and the Pearson product moment correlation coefficient for ITS1-SSCP and AFLP analysis, respectively. Cluster analysis was performed with the un-weighted pair-group method using average linkages (UPGMA). Reliability of the cluster analysis was determined by calculating the cophenetic values, representing the correlation between calculated similarities and dendrogram-derived similarities. Sequence handling, calculation of similarity values, cluster, and multi-dimensional scaling analysis used BioNumerics®. Sequence quality was enhanced with ABI long trace software (http://www.nucleics.com/longtrace-sequencing/) and assembled with Batch Sequence Assembly plug-in (BioNumerics®). Multiple alignment and cluster analysis of sequences used the Alignment & Mutation Analysis tool in BioNumerics®. Similarity matrices, distances (Jukes and Cantor [10] correction), neighbor-joining [11], maximum parsimony, and maximum likelihood trees were generated using BioNumerics®.

3. Results

3.1. ITS1-SSCP typing

The thirty-two worms were fingerprinted using ITS1-SSCP. Amplification with primers ITS1 and E5.8S yielded the expected 550 bp fragment and, after *Taq*I digestion, two restriction fragments of 400 and 150 bp were observed in all individuals (data not shown). To determine if sequence polymorphisms were present in the ITS1 fragments but not detected by standard electrophoresis, fragments were resolved by SSCP and subsequently revealed 9 distinct SSCP profiles of 6–9 bands each (Fig. 1). Profiles differing in at least one band were considered distinct types. Note that each denatured double-stranded fragment in an SSCP profile was represented in the gel by several bands because as the single-strands migrate through the gel the same strand of DNA may adopt several conformations not necessarily of equal staining intensity [12]. Unique ITS1-SSCP types were defined at 100% similarity, and minimum percent similarity among ITS1-SSCP types was 55%. Six ITS1-SSCP types (Types 1, 2, 5–8) were each represented by a single *N. klawei* individual; whereas, ITS1-SSCP Types 3, 4 and 9 grouped 10, 13, and 3 *N. klawei* individuals collected from 6, 9, and 2 yellowfin tuna, respectively (cophenetic values >80) (Table 1). In two instances (fish numbers 1 and 7), two *N. klawei* collected from each of the right and left nasal cavities shared ITS1-SSCP types 3 and 4, respectively (Table 1). Six of nine fish (fish numbers 1, 4, 6, 7–9) had a corresponding nasal cavity harboring an

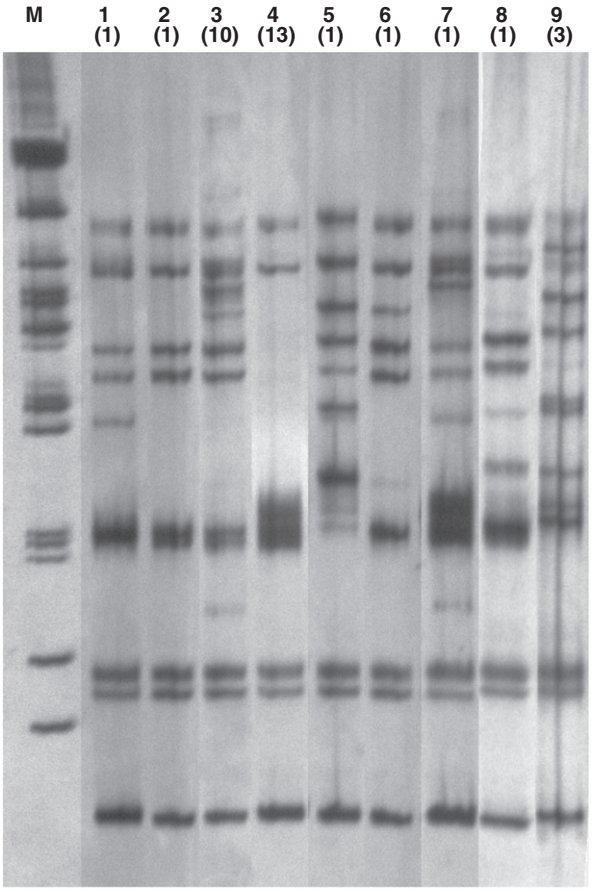

Fig. 1. ITS1-SSCP types (lanes 1–9) obtained from thirty-two *Nasicola klawei* collected from nine yellowfin tuna, *Thunnus albacares*, in the Gulf of Mexico. Bracketed numbers equal the number of *N. klawei* sharing an ITS1-SSCP type. M = molecular weight marker.

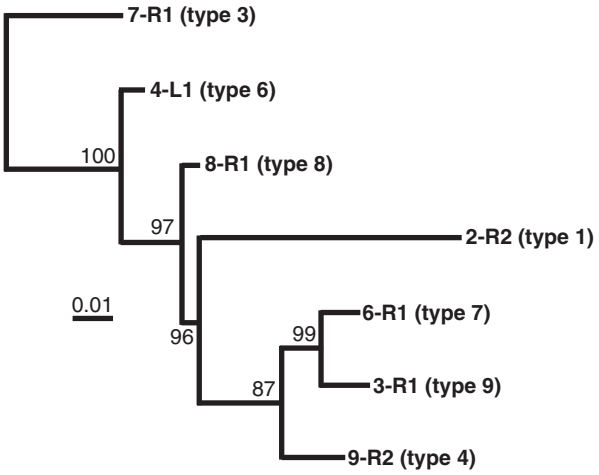

Fig. 2. Maximum parsimony tree based on ITS1 sequences of seven of nine ITS1-SSCP types, which are indicated in brackets aside each terminal code, obtained from a sample of thirty-two individual *Nasicola klawei* collected from nine yellowfin tuna, *Thunnus albacares*, in the Gulf of Mexico. The tree is a consensus bootstrap tree based on 1000 re-sampled trees; bootstrap values indicated on branches. Terminal codes denote host number–right (R) or left (L) nasal cavity–*N. klawei* specimen number. Scale bar represents 1% sequence divergence.

N. klawei of the same ITS1-SSCP type (Table 1). None of the nine analyzed *N. klawei* infrapopulations was comprised of a single ITS1-SSCP type, but two *N. klawei* from the same infrapopulation were of the same ITS1-SSCP type in nine instances. Only ITS1-SSCP Type 4 was found in at least one *N. klawei* from each yellowfin tuna sampled (Table 1), indicating that not all ITS1-SSCP types are equally distributed among all *N. klawei* sampled. The ITS1-SSCP discrimination index was 0.75 [13].

3.2. ITS1 sequence

A 420 bp ITS1 sequence was obtained from each selected specimen representing Types 1, 3, 4, 6–9 (Table 1, Fig. 1). Nucleotide variation among these sequences ranged from 1 to 11 of 420 (0.2–2.6%) total nucleotides. Phylogenetic analysis of all ITS1 sequences revealed concordant tree topologies regardless of the method used (neighbor joining, maximum parsimony, or maximum likelihood), and, except for the 6R-1 clade (Fig. 2), nodes were supported by high (>87) bootstrap values.

3.3. AFLP

Twenty-three worms were analyzed by AFLP (Fig. 3); the remaining 9 samples failed to yield the high quality (intact, non-fragmented) DNA needed for AFLP. All primer-pair combinations yielded complex AFLP patterns consisting of 80 to 110 bands of 50–700 bp (Fig. 3). All AFLP profiles clustered at 90% similarity or lower, indicating that each *N. klawei* was significantly distinguishable from others. All individuals clustered at 55% similarity or higher. The discrimination index for the AFLP as a typing system for *N. klawei* was 1 [13]. Primer-pair combination E.CTA/M.ACC was selected for further analysis based on band coverage; it was the combination that produced the broader molecular weight range of bands. AFLP depicted no *N. klawei* infrapopulation as monophyletic (Fig. 3). In only two incidences were worms from the same infrapopulation grouped as sister taxa, i.e., 3-L2 + 3-R1 (83%) and 9-L1 + 9-R2 (90%) (each boxed in Fig. 3).

3.4. 28S rDNA sequence

The 5′ terminal 28S rDNA (D1–D3 domains) was amplified from 4 *N. klawei* with different ITS1-SSCP types and dissimilar AFLP profiles. The expected ~900 bp amplicon was obtained in all cases and no sequence heterogeneity was found among partial 28S rDNA sequences (Table 1). These sequences were identical (100% maximum similarity) to *N. klawei* sequences in GenBank (FJ972003).

4. Discussion

4.1. ITS1-SSCP intraspecific variability

The SSCP technique used in our ITS1 analysis ensures that intraspecific variation is represented in sequencing analysis during the screening of large population samples. The method is relatively inexpensive and rapid [14] as it identifies unique DNA samples so that only representative types need be sequenced. Although widely applied in medical diagnosis [15], this method is presently vastly underexplored among pelagic marine fish parasites.

Relatively few molecular markers have been explored in monogeneans, but ITS1 so far shows the greatest documented variability [16]. ITS1 and ITS2 of the ectoparasitic *Gyrodactylus salaris* and congeners have been scrutinized for purposes of taxonomic identification [17], phylogenetic reconstruction [18], and population dynamics [19], and differences in those ITS1 sequences routinely are used for species diagnosis [20]. The ITS1 of *N. klawei* varied by 1–11 of 420 bp (0.2–2.6%) (Figs. 1, 2), suggesting that this marker should be used with caution in delineating at least capsaline species and especially *Nasicola* spp. from different ocean basins. Forgoing sympatric speciation and given that we studied worms from the same site of infection (nasal cavity), in a single host species (yellowfin tuna), and from the same geographic locality (Gulf of Mexico), it is unlikely that a species complex of *Nasicola* was

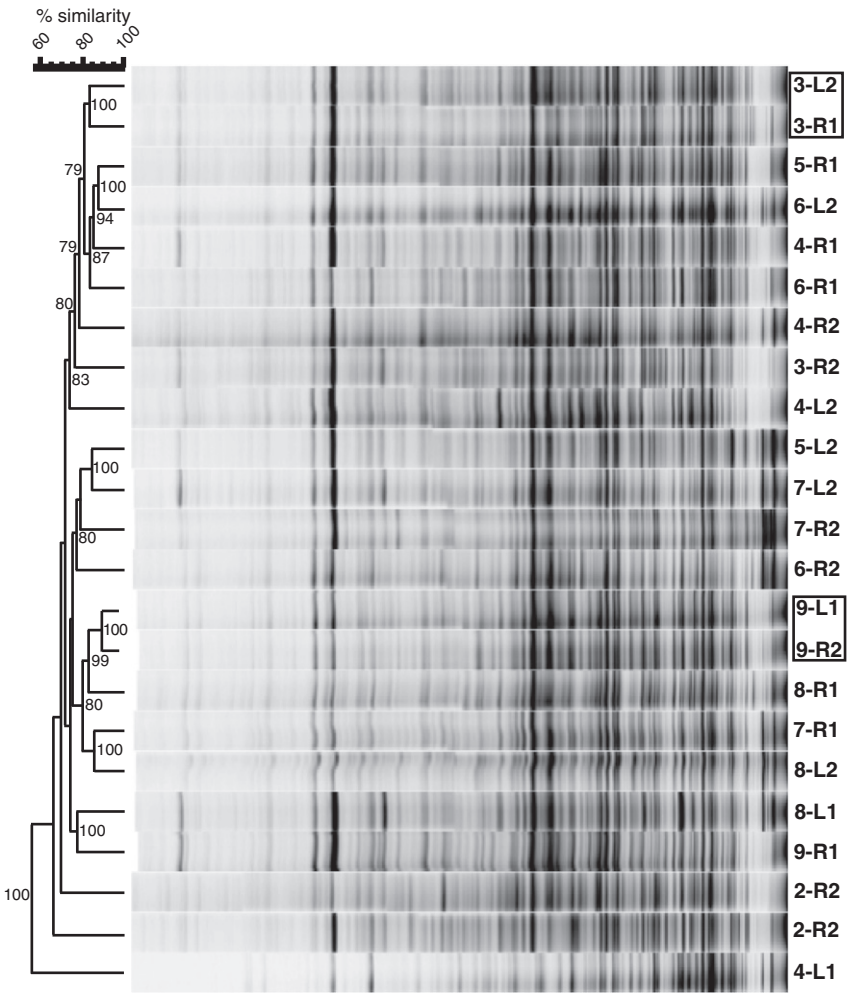

Fig. 3. Dendrogram based on AFLP patterns from twenty-three *Nasicola klawei* collected from nine yellowfin tuna, *Thunnus albacares*, in the Gulf of Mexico. The tree was derived by UPGMA cluster analysis of AFLP profiles. Levels of linkage are expressed as percent of similarity based on the Pearson product–moment coefficient. Cophenetic values >75 are displayed on each node. Terminal codes denote: host number–right (R) or left (L) nasal cavity–*N. klawei* specimen number.

represented in our sample. However, such hidden taxonomic diversity, if present, would obviously not alter our conclusions about the studied infrapopulations.

4.2. AFLP intraspecific variability

AFLP analysis [21–23] is a highly sensitive technique that defines genome-wide DNA polymorphisms used to identify species or subspecies [24] and strains [9] as well as to infer phylogeny [25] and infrapopulation genetic variability [26]. It is a powerful method when used on taxa for which no or few sequence data exist because it reveals a large number of putatively independent nucleotide polymorphisms in a genome without prior knowledge of the genome's sequence. This universal fingerprinting method has proved useful for population genetics of parasitic nematodes [27] and *Gyrodactylus* spp. [20], but prior to the present report AFLP had not been applied to any other ectoparasitic flatworm in fish. Herein, AFLP analysis, like that of ITS1-SSCP, suggested that genetic relatedness among individuals of *N. klawei* was not structured according to host individual, i.e., no infrapopulation was monophyletic by AFLP. AFLPs are considered dominant markers (they cannot differentiate between the heterozygotic or homozygotic state of a locus), which results in low discrimination between samples from within an inbred population. All AFLP profiles among our samples were unique, strongly suggesting that these infrapopulations were not the result of inbreeding [28] or of self-fertilization and subsequent autoinfection from one nasal cavity to the other.

4.3. Life history

No capsaline life cycle is published but it probably follows the pattern typically observed in other monogeneans. The hermaphroditic adult copulates with itself or another individual before producing an egg that encloses a single, microscopic larva (oncomiracidium) that hatches and autoinfects or enters the plankton as an embryonated egg or ciliated oncomiracidium [29]. Capsalines infect tunas (Scombridae), billfishes (Istiophoridae and Xiphiidae), and sunfishes (Molidae), and colonization of these hosts seems challenging for a minute planktonic egg or oncomiracidium. For example, Stunkard [30] stated that for *N. klawei*, "*infection of fast-swimming, wide-ranging fishes would appear difficult*." Although the extra-uterine eggs of *N. klawei* had not been observed at the time, Stunkard correctly predicted that they had elongated filaments, based on the notion that projecting filaments might facilitate transmission by becoming entangled in a tuna's gill. This, however, seems likely only for horizontal dispersal, since eggs ejected from the nasal cavity would not be inhaled and pass over the gill. Noteworthy too is that those same filaments may be adaptive for entangling in olfactory lamellae as a mechanism to facilitate egg hatching within the nasal cavity. Forty-two years after Stunkard's prediction, Kohn et al. [31] detailed the egg of *N. klawei* that indeed has four filaments, which probably reduces sinking and therefore could increase the likelihood that hatching will occur in the epipelagic zone. Subsequently, Chisholm and Whittington [32] detailed the ciliated oncomiracidium of *N. klawei* as having six "*large refringent (lipid?) droplets*" within its body. They

speculated that these droplets, which are lacking in at least a few non-capsaline oncomiracidia [33], comprised an energy store capable of sustaining the oncomiracidium during its potentially long residence time in the plankton prior to host colonization. We think those same droplets, like the egg filaments, may also reduce sinking. The schooling behavior of tunas may also facilitate such horizontal infection.

Two simple scenarios exist for monogenean dispersal: (a) colonization of a potential host other than that of its parent(s) (=horizontal dispersal; lengthy residency time in water column; intuitively lower probability of successfully finding the appropriate host) or (b) colonization of the same fish infected by its parent(s) (=autoinfection; brief or no residency time in water column; intuitively higher probability of finding host). For susceptible, crowded fishes that are confined (such as in aquaculture or aquarium settings) or tightly schooling, "success" in either scenario seems equally probable because of the close proximity of potential hosts. However, for solitary or schooling, free-ranging epipelagic fish the intuitive probability of a minute oncomiracidium successfully finding its specific host seems lower. Considering these scenarios in light of ITS1-SSCP analysis, AFLP analysis, and DNA sequencing of *N. klawei* population structure, we hypothesized that parasite infrapopulation paraphyly would unequivocally support the horizontal dispersal scenario; whereas, infrapopulation monophyly would signal, equivocally, that (i) sibling oncomiracidia had infected the host by horizontal dispersal or (ii) a single-oncomiracidium had colonized the host and reproduced via self-fertilization with its progeny directly infecting that host (autoinfection).

Our ITS1-SSCP and AFLP results together (Figs. 1–3) show appreciable levels of intraspecific genetic diversity among *N. klawei* and simultaneously no genetic exclusivity recognizable as *N. klawei* infrapopulation monophyly (Fig. 3). This led us to conclude that multiple collisions between planktonic oncomiracidia and yellowfin tuna best explain the origin of each infrapopulation. The alternative scenario, i.e., an oncomiracidium colonizing a tuna before self-fertilization and propagation of auto-infecting oncomiracidia, is not well-supported by the present data.

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References

[1] Combes C. Parasitism: the ecology and evolution of intimate interactions. Chicago and London: The University of Chicago Press; 2001.
[2] Littlewood DTJ. Marine parasites and the tree of life. In: Rohde K, editor. Marine parasitology. Collingwood: CAB International Publishing and CSIRO Publishing; 2005. p. 6–10.
[3] Whittington ID. Monogenea Monopisthocotylea (ectoparasitic flukes). In: Rohde K, editor. Marine parasitology. Collingwood: CAB International Publishing and CSIRO Publishing; 2005. p. 63–72.
[4] Archie EA, Ezenwa VO. Population genetic structure and history of a generalist parasite infecting multiple sympatric host species. Int J Parasitol 2011;41:89–98.
[5] Huyse T, Poulin R, Théron A. Speciation in parasites: a population genetics approach. Trends Parasitol 2005;21:469–75.
[6] Chisholm LA, Whittington ID. Review of the Capsalinae (Monogenea: Capsalidae). Zootaxa 2007;1559:1–30.
[7] Li A-X, Wu X-Y, Ding X-J, Lin R-QM, Xie -Q, Lun Z-R, Zhu X-Q. PCR-SSCP as a molecular tool for the identification of Benedeniinae (Monogenea:Capsalidae) from marine fish. Mol Cell Probes 2005;19:35–9.
[8] Olivares-Fuster O, Shoemaker CA, Klesius PH, Arias CR. Molecular typing of *Flavobacterium columnare* isolates by single stranded conformation polymorphism analysis. FEMS Microbiol Lett 2006;269:63–9.
[9] Arias CR, Welker TL, Shoemaker CA, Abernathy JW, Klesius PH. Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish. J Appl Microbiol 2004;97:421–8.
[10] Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press; 1969. p. 21–132.
[11] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.
[12] Schmalenberger A, Schwieger F, Tebbe CC. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl Environ Microbiol 2001;67:3,557–63.
[13] Hunter PR. Reproducibility and indices of discriminatory power of microbial typing methods. J Clin Microbiol 1990;28:1,903–5.
[14] Sunnucks PA, Wilson CC, Beheregaray LB, Zenger K, French J, Taylor AC. SSCP is not so difficult: the application and utility of single-strand conformation polymorphism in evolutionary biology and molecular ecology. Mol Ecol 2000;9:1,699–710.
[15] Dong Y, Zhu H. Single-strand conformational polymorphism analysis. Methods Mol Med 2005;108:149–58.
[16] Olson PD, Tkach VV. Advances and trends in the molecular systematics of the parasitic Platyhelminthes. Adv Parasitol 2005;60:165–243.
[17] Huyse T, Vockaert FAM. Identification of a host-associated species complex using molecular and morphometric analyses, with the description of *Gyrodactylus rugiensoides* n. sp. (Gyrodactylidae, Monogenea). Int J Parasitol 2002;32:907–19.
[18] Zietara MS, Huyse T, Lumme J, Volckaert FA. Deep divergence among subgenera of *Gyrodactylus* inferred from rDNA ITS region. Parasitology 2002;124:39–42.
[19] Meinilä M, Kuusela J, Zietara M, Lumme J. Initial steps in speciation by geographical isolation and host switch in the salmonid pathogen *Gyrodactylus salaris* (Monogenea). Int J Parasitol 2004;34:515–26.
[20] Bakke TA, Cable J, Harris PD. The biology of gyrodactylid monogeneans: the "Russian-doll killers". Adv Parasitol 2007;64:161–376.
[21] Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuper M, Zabeau M. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 1995;23:4,407–14.
[22] Liu Z. Amplified fragment length polymorphism (AFLP). In: Liu X, editor. Aquaculture genome technologies. Oxford: Blackwell Publishing Ltd.; 2007. p. 29–42.
[23] Graves J. Deeper AFLPs. Heredity 2009;103:99.
[24] Gwo J, Hsu T, Lin K, Chou Y. Genetic relationship among four subspecies of cherry salmon (*Oncorhynchus masou*) inferred using AFLP. Mol Phylogenet Evol 2008;48:776–81.
[25] Mendelson TC, Wong MK. AFLP phylogeny of the snubnose darters and allies (Percidae: *Etheostoma*) provides resolution across multiple levels of divergence. Mol Phylogenet Evol 2010;57:1,253–9.
[26] Techaprasan J, Klinbunga S, Jenjittikul T. Genetic relationships and species authentication of *Boesenbergia* (Zingiberaceae) in Thailand based on AFLP and SSCP analyses. Biochem Syst Ecol 2008;36:408–16.
[27] Gruijter JM, Polderman AM, Dijkshoorn L, Roberts H, Ziem J, Kunwar CB, Gasser RB. AFLP fingerprinting for the analysis of genetic diversity within *Necator americanus*. Mol Cell Probes 2006;20:317–21.
[28] Nejsum P, Frydenberg J, Roepstorff A, Davis Parker Jr E. Population structure of *Ascaris suum* (Nematoda) among domestic swine in Denmark as measured by whole genome DNA fingerprinting. Hereditas 2005;142:7–14.
[29] Whittington ID, Chisholm LA. Diseases caused by Monogenea. In: Eiras JC, Segner H, Wahli T, Kapoor BG, editors. Fish diseases, Vol. 2. Enfield: Science Publishers; 2008. p. 683–816.
[30] Stunkard HW. *Caballerocotyla klawei* sp. n., a monogenetic trematode from the nasal capsule of *Neothunnus macropterus*. J Parasitol 1962;48:883–90.
[31] Kohn A, Baptista-Farias MF, dos Santos AL, Gibson DI. A new species of *Nasicola* Yamaguti, 1968 (Monogenea: Capsalidae) from the nasal cavities of *Thunnus obesus* and a redescription of *N. klawei* (Stunkard, 1962) from *T. albacares* off Brazil. Syst Parasitol 2004;57:51–8.
[32] Chisholm LA, Whittington ID. The oncomiracidium of *Nasicola klawei* (Monogenea: Capsalidae: Capsalinae). Parasitol Res 2007;100:467–71.
[33] Kearn GC. The oncomiracidium of *Capsala martinieri*, a monogenean parasite of the sun fish (*Mola mola*). Parasitology 1963;53:449–53.