Biol Invasions
DOI 10.1007/s10530-011-0111-y

ORIGINAL PAPER

Genetic relationships of the marine invasive crab parasite *Loxothylacus panopaei*: an analysis of DNA sequence variation, host specificity, and distributional range

Inken Kruse · Matthew P. Hare · Anson H. Hines

Received: 23 June 2010 / Accepted: 16 September 2011

Abstract Host specificity is a key variable of the niche breath of parasites that can be an important determinant of a parasite's ability to invade new areas. There is increasing evidence that many parasite species may comprise a variety of genetically variable lineages, which differ in host specificity and geographic range. In this study, we (1) explored the extent of diversity in the invasive parasitic barnacle *Loxothylacus panopaei* (Rhizocephala) infecting mud crabs (2) examined the geographic origin for the invasive lineage and (3) assessed if further southward spread of the parasite may be impeded. Along the US Atlantic coast, *L. panopaei* infects different hosts in its invaded range (Chesapeake Bay to north of Cape Canaveral) compared to one portion of the native range in Southeast Florida. This difference was reflected in genetic lineages on two independent loci, mitochondrial cytochrome oxidase I and nuclear cytochrome c. Both loci were concordant in that they showed one lineage infecting crabs of the genus *Panopeus* in the native range and one lineage infecting *Eurypanopeus depressus* and *Rhithropanopeus harrisii* hosts in the invaded range and in the Gulf of Mexico, thus indicating Gulf of Mexico populations as the most likely source of introduction into Chesapeake Bay. Interestingly, the nuclear marker resolved an additional lineage of parasites infecting panopeid hosts in the native range. All three parasite lineages were well supported, but a decision about species status must await further analyses. Since its introduction in the 1960s, the invasive *L. panopaei* lineage has expanded its range southward along the US Atlantic coast, now almost reaching the northern limit of native *Panopeus*-infecting lineages at Cape Canaveral, Florida. We hypothesized that parasite-free *E. depressus* in Southeast Florida, living in sympatry with infected panopeid populations, might be resistant to infection by the invasive lineage. Our infection experiments rejected this hypothesis, suggesting that any impediment to further southward range expansion might be expected from temperature regimes of the subtropical zoogeographic region south of Cape Canaveral.

Keywords Phylogeography · Mitochondrial introgression · Nuclear DNA · Rhizocephala · Sacculinidae

I. Kruse
Smithsonian Marine Station, Ft. Pierce, FL 34949, USA

Present Address:
I. Kruse (✉)
Benthic Ecology, Leibniz-Institut für Meereswissenschaften, IFM-GEOMAR, Düsternbrooker Weg 20, 24105 Kiel, Germany
e-mail: ikruse@ifm-geomar.de

M. P. Hare
Department of Natural Resources, Cornell University, 208 Fernow Hall, Ithaca, NY, USA

A. H. Hines
Smithsonian Environmental Research Center, 617 Contees Wharf Road, Edgewater, MD 21037, USA

Published online: 27 September 2011

Introduction

Host specificity is one of the most important characteristics of a parasite and is considered as equivalent to resource specialization in free living organisms (e.g. Poulin 2007). Generalist parasitic species that infect multiple host species may have the ability to add a new host species or populations to their repertoire of potential hosts, whereas locally adapted specialist parasites may be limited by availability of a few suitable hosts. Thus, host specificity can determine whether a parasite has the potential to expand its established range, or whether a parasite can become established and spread following its introduction to a new geographical area.

According to the niche breadth hypothesis (Brown 1984, 1995), generalists have broader geographic ranges than specialists. A geographical perspective has been lacking from most previous investigations of parasite host specificity (Poulin 2007, p. 69), but at least for fleas the niche breadth hypothesis was supported by testing the relationship of host specificity and geographic range on a global scale (Krasnov et al. 2005).

However, assessing geographic range and host specificity can be confounded by the presence of cryptic lineages of parasites, which when unrecognized suggest broader host specificity and geographic range than for their specific variants (Miura et al. 2005, 2006; Poulin 2007 and references therein, p. 42; Poulin and Keeney 2008). Cryptic lineages are common in parasitic groups, because they are often under-studied, are involved in very specialized interspecific interactions, and/or lack morphological distinctness due to selection on behavioural and physiological characters rather than on morphological characters, which are traditionally used in taxonomy (Bickford et al. 2007). Marine parasites may be especially prone to unrecognized diversity because marine organisms often rely on chemical signals for key adaptations, rather than visually distinct morphological structures (Knowlton 2000).

In this study, we further examined the genetic diversity, geographic distribution and host specificity of the parasitic barnacle *Loxothylacus panopaei* (Rhizocephala) that infects estuarine xanthoid mud crabs, the most common crabs inhabiting oyster reefs along the east coast of the United States (Williams 1984). The life-cycle of rhizocephalan barnacles starts when female larvae infect recently molted crabs and proliferate internally, producing a small virgin externa protruding from the crab abdomen. Male larvae then enter the externa and fertilize eggs (Høeg and Lützen 1995), which ultimately leads to a full externa similar in gross appearance to a crab egg mass, from which larvae are released at biweekly intervals. As a result of this life history, the host crabs are castrated, potentially having strong demographic effects on the host population (Kuris 1974; Antonovics 2009).

The native range of *L. panopaei* included Southeast Florida, the Gulf of Mexico (GOM) and Caribbean waters (Boschma 1955). In the 1960s this parasite was introduced into Chesapeake Bay, presumably via infected host crabs associated with oyster transports from the GOM (Van Engel et al. 1966), where it subsequently reached infection levels of up to 93% (Hines et al. 1997; Kruse and Hare 2007). After a period of expansion within Chesapeake Bay, *L. panopaei* expanded its range southward along the US. Atlantic coast down to Cape Canaveral over 40 years, most recently striding ahead by 170 km in 1 year (Kruse and Hare 2007). In this study, we sampled the Cape Canaveral region at finer grid in order to better define the expanding front of the spreading invasion (Figs. 1, 2).

A previous study showed that this parasite infects a different set of host species in its introduced range at the US. Atlantic coast compared to the native range (Hines et al. 1997). In the Chesapeake Bay and southward to Jacksonville, Florida, *Eurypanopeus depressus, Rhithropanopeus harrisii* and *Dispanopeus sayi* hosts were infected, while in Southeast Florida *L. panopaei* appeared restricted to crabs of the genus *Panopeus*, and had very low to no prevalence in *E. depressus* (1 infection in 1,358 crabs; Hines et al. 1997). Based on mitochondrial DNA, the genetic form of the parasite which infects crabs of the genus *Panopeus* in Southeast Florida appears to have a northern geographic limit at Cape Canaveral (Kruse and Hare 2007). In Southeast Florida, Kruse and Hare (2007) did not find *L. panopaei* in *E. depressus* hosts despite high abundances of this mud crab on the same oyster beds where crabs of the genus *Panopeus* had a 16% infection occurrence. This may reflect different host specificities between native and invasive parasite lineages, or resistance to infection among Southeast Florida *E. depressus* populations. If the latter is true with respect to the native *L. panopaei* lineage, then possibly these crab populations are also resistant to infection by the southward invading lineage. Several

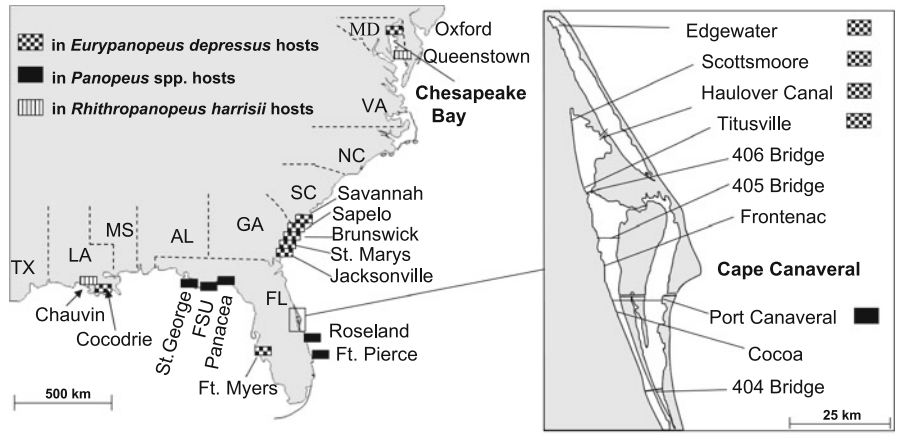

Fig. 1 Atlantic and Gulf of Mexico (GOM) sites sampled in 2006. *Rectangles* indicate presence of the parasite *Loxothylacus panopaei* and pattern inside *rectangles* specifies crab host species (see legend in *upper left corner*)

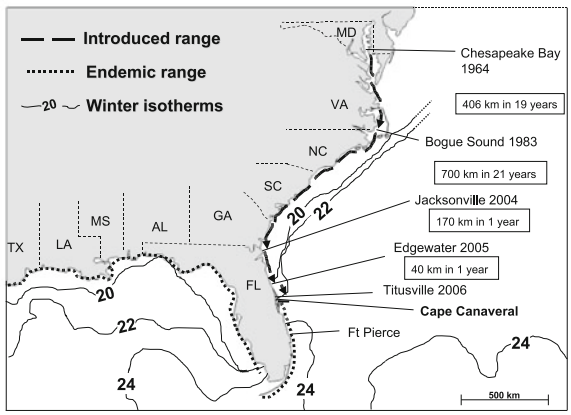

Fig. 2 Introduced and native ranges of *Loxothylacus panopaei*, range expansion since introduction into Chesapeake Bay and winter isotherms of sea surface temperatures (http://www.osdpd.noaa.gov)

species which are broadly dispersed across the zoogeographic boundary around Cape Canaveral (Briggs 1974), as is *E. depressus,* show phylogenetic disjunctions centred on this Atlantic temperate tropical transition zone off east-central Florida (Saunders et al. 1986; Sarver et al. 1992; Hare and Avise 1996; Lee and Ó Foighil 2004). Thus, *E. depressus* south of Cape Canaveral may share a different co-evolutionary history with the parasite, represent a different lineage than *E. depressus* north of Cape Canaveral and may be insusceptible to the invasive lineage of *L. panopaei* in infection experiments.

Here, we test for possible genetic differences among parasite populations in relation to geography and host specificity. Moreover, in 2004 and 2005, Kruse and Hare (2007) did not find *L. panopaei* infections of *E. depressus* and *R. harrisii* hosts from the GOM to test if this area was the source of the Chesapeake Bay introduction, as had been suspected by Van Engel et al. (1966). Thus, the specific goals of this follow up study were to: (1) measure geographic distribution and prevalence of the parasite *L. panopaei*; (2) explore associations between the parasite's genetic lineages with host specificity in the GOM; (3) test the hypothesis that the GOM was the source of the Chesapeake Bay introduction; and (4) assess if further southward spread of the parasite may be impeded at the biogeographic barrier at Cape Canaveral due to host resistance. For testing host resistance, we examined if Southeast Florida populations of the host species *E. depressus* and *R. harrisii* are prone to experimental infection by the invasive parasite from north of Cape Canaveral.

Methods

Sampling

A total of 4,011 xanthoid crabs, 5 mm or larger, were sampled by hand during 2006 from oyster beds at 19 sites along the Florida Atlantic coast (from Edgewater near Cape Canaveral to Fort Pierce) and in the Gulf of Mexico (GOM) (Tables 1, 2; Fig. 1). The crabs depend on patchily distributed oyster shell or rock rubble habitats and not all sampled sites offered these habitats in abundance. For this reason, sample sizes of crabs varied strongly between sites. All crabs were preserved in 95% ethanol. Prevalence of the parasite was determined as percentage of crabs with externae (i.e. full externae and virgin externae).

Table 1 Site coordinates of *Loxothylacus panopaei* sampling and sequenced individuals for mitochondrial (COI) and nuclear (CytC) loci. For further details on samplings, see Table 2

Species	Site	Coordinates	mtDNA (COI) individual ID-Nr.	nDNA (CytC) individual ID-Nr.
L. panopaei	Chesapeake, Oxford	N38 41; W76 10	1, 2, 3, 4	1, 3, 4, 6, 7, 10
	Chesapeake, Queenstown	N37 40; W76 29	1, 2, 5	2, 3, 4, 10
	Savannah	N31 57.07; W81 05.89	1, 2, 3, 4	
	Sapelo	N31 23; W81 16	1, 3, 4, 6	4, 5, 6
	Brunswick	N31 09.24; W81 34.20	1, 2, 3, 4	2, 3, 4, 6, 8, 10
	St. Marys	N30 43.20; W81 32.83	1, 2, 3, 4, 5, 6, 7, 8	1, 2, 5, 6, 9
	Jacksonville	N30 23.83; W81 26.14	1, 2, 3, 4, 5, 6, 7	3, 9, 12, 14, 18
	Egdewater	N28 59.62, W80 54.23		
	Scottsmoore	N28 46.24, W80 50.73		
	Haulover Canal	N28 43.99, W80 45.42		
	Titusville	N28 37.92, W80 49.13		
	406 Bridge	N28 37.47, W80 47.58		
	405 Bridge	N28 31.62, W80 44.50		
	Frontenac	N28 27.59, W80 45.68		
	Port Canaveral	N28 24.59, W80 37.99		
	Cocoa	N28 16.31, W80 41.28		
	404 Bridge	N28 12.46, W80 39.79		
	Roseland	N27 50.71; W80 29.01	2	
	Ft. Pierce	N27 29.91, W80 18.72	1, 5, 8, 9, 10, 14, 15, 16	5, 6, 8, 9, 10, 12, 13,14, 15, 17, 18
	Ft. Myers	N26 26 41, W81 52 08	1, 2, 3	1, 2, 3
	Panacea	N29 54.89, W84 30.81	1, 2, 3, 4, 5, 7, 8	1, 3, 6
	FSU Marine Lab	N29 41.59, W84 47.16	1	1
	St. George Isl.	N30 01.48, W84 22.07		1
	Dauphin Island—Saltmarsh	N30 15.24, W88 05.01		
	Dauphin Island—Airport	N30 15.50, W88 07.39		
	Cocodrie	N29 15.24, W90 39.82	1, 2, 3, 4	1, 2, 3, 4
	Chauvin LakeQuitman	N29 19.97, W90 38.55		1
	Chauvin	N29 23.15, W90 37.20	1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 14, 15	5, 6, 7, 10, 11, 12, 14, 15
L. texanus	Panacea	N30 01, W084 23	1, 2	
	Tampa 1	N27 46.26, W082 26.97	1	
	Tampa 2	N27 43.30, W082 44.09	2	

Genotyping

Individual *L. panopaei* found in the 4,011 crabs (see above) and also at one site near Fort Myers ('Estero Bay' samples from 2002, see Tolley et al. 2006), were analyzed genetically using the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) gene and the nuclear cytochrome c (CytC) marker (Table 2). COI sequences from a previous study from 2004 and 2005 samples also were included in the analyses (Kruse and Hare 2007), and parasites from the same sites additionally were analyzed for the CytC locus. DNA extraction and genotyping methods using the customized COI marker followed Kruse and Hare (2007). The exon-primed, intron-crossing (EPIC) primers for CytC introns (Palumbi 1996) were used as the nuclear marker: CytC-C-5′ 5′-AAG TGT GCY CAR TGC CAC AC-3′ and CytC-B-3′ 5′-CAT CTT

Table 2 Prevalence of *Loxothylacus panopaei* infections in three crab host genera in 2006 at Florida Atlantic and Gulf of Mexico sites; sites arranged from north to south (Atlantic) and west into the Gulf of Mexico. The italicised values are the three parasite-free sites between the expanding front (at Titusville) and the native northern range limit (Port Canaveral)

	Date	*Eurypanopeus depressus*		*Panopeus* spp.		*Rhithropanopeus harrisii*	
		N	% Infection	*N*	% Infection	*N*	% Infection
Atlantic coast							
Egdewater	17-Apr-06	49	82	45	0		
	11-May-06	34	53	72	0		
Scottsmoore	20-Apr-06	61	2	1	0		
Haulover Canal	18-Apr-06	113	11	72	0		
Titusville	20-Apr-06	89	9	0	0		
	27-Jun-06	39	8	22	0		
406 Bridge	*18-Apr-06*	*106*	*0*	*1*	*0*		
405 Bridge	*02-May-06*	*218*	*0*				
Frontenac	*11-May-06*	*182*	*0*	*2*	*0*	*17*	*0*
Port Canaveral	02-May-06	29	0	4	100		
	27-Jun-06	17	0	14	86		
Cocoa	11-May-06	34	0			19	0
404 Brigde	11-May-06	109	0			13	0
Ft. Pierce	24-May-06	163	0	55	29		
	21-Jun-06	108	0	54	19		
Gulf of Mexico							
Ft. Myers[a]	June 2002	2,648[a]	1[a]				
Panacea	02-Apr-06	127	0	114	3		
FSU Marine Lab	31-Mar-06	113	0	187	1		
St. George Isl.	01-Apr-06	298	0	13	8		
Dauphin Island—Saltmarsh	04-Apr-06	372	0	12	0		
Dauphin Island—airport	05-Apr-06	459	0	10	0		
Cocodrie	07-Apr-06	447	1				
Chauvin-LakeQuitman	07-Apr-06					8	50
Chauvin	07-Apr-06					109	14
Sum per crab species		3,167		678		166	
Sum all crab species						4,011	

[a] Sampling from 2002 (see Tolley et al. 2006) not included in sums above

GGT GCC GGG GAT GTA TTT CTT-3′. From these sequences, we designed more specific primers: CytC-Lxpa-R 5′-CAA TGT TCA CAG GGG TAG GTG-3′ and CytC-Lxpa-L 5′-TCA AAA CGC TCA CCA CAA AC-3′. The PCR conditions used to amplify the CytC fragment with the latter primers in a 20-µl reaction were: 1× *Taq* PCR buffer, 0.6 units *Taq* (both Invitrogen, Carlsbad, California), 1.0 µl DNA template, 20 mg/µl BSA, 5 mM $MgCl_2$, 0.2 µM each primer, and 0.25 mM dNTPs. These reactions were run on a MJ Peltier PTC-255 thermocycler (MJ Research, Watertown, Massachusetts). Temperature cycling started with 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 1 min at 65°C and 45 s at 72°C, and finished by a final extension step of 10 min at 72°C.

For sequencing, all templates were cleaned with the use of shrimp alkaline phosphatase and exonuclease. Sequence reactions were performed in 10 µl volumes, using 1–3 µl of the cleaned sample, 33 mM of forward or reverse primer, 2 µl of ABI BigDye Terminator ver. 3.1 and 1.5 µl of 5× buffer (both Applied

Biosystems). After cleanup of the sequence reactions using gel filtration cartridges from Edge Biosystems, the sequences were analyzed with a ABI 3100 automatic sequencer at the Smithsonian Institution Laboratories of Analytical Biology, Suitland, MD, USA. Sequence chromatograms for forward and reverse strands were aligned in Sequencher 4.0 to manually edit ambiguous base calls and assign IUPAC-IUB nucleotide codes. Unique sequences were deposited in GenBank with accession numbers HQ665506-HQ665548 (CytC) and HQ848063-HQ848078 (COI) (Table 1). *Loxothylacus texanus* samples (the congeneric parasite infecting the portunid crab *Callinectes sapidus* in the GOM) were used as an outgroup in the COI tree, but all nuclear CytC primers, both universal and custom, failed to amplify *L. texanus.*

Data analysis

Chi-square tests of homogeneity of base frequencies across clades were conducted with PAUP*, v4.0b10 (Swofford 2001) to estimate the frequency distribution of observed number of substitutional changes per character for both loci. No significant differences for both loci were revealed. DNA polymorphism and divergence statistics were calculated using DAMBE Version XXX (Xia and Xie 2001), with nucleotide diversity based on Nei (1987). COI sequences were translated using MEGA version 4 (Tamura et al. 2007). The COI sequences had on average less than 1% missing data and no gaps (indels), whereas the CytC sequence alignment had 14 gaps (4.8% of the alignment) that were treated as missing data. Each locus was analysed separately with all sequences included for calculation of diversity indices and with identical haplotypes (aligned using Sequencher 4.0) collapsed for phylogenetic analyses. Since this resulted in a single haplotype for clade P in the COI dataset, two copies of this haplotype were used in maximum likelihood, maximum parsimony and bayesian analyses to get bootstrap support and posterior probabilities for this clade; Figs. 3 and 4 show these support values for a single haplotype for clade P.

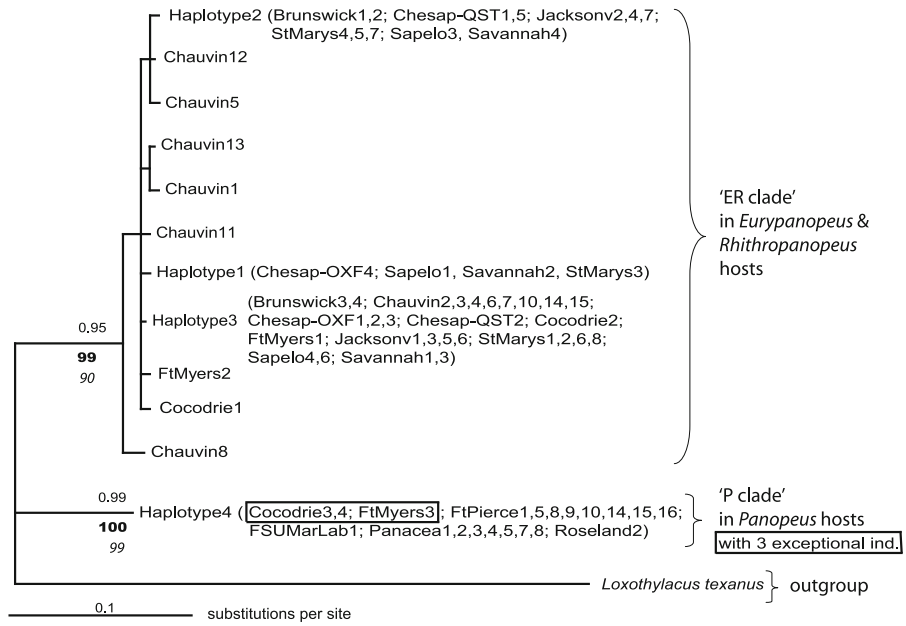

Fig. 3 Bayesian tree for *Loxothylacus panopaei* on the mitochondrial locus COI with *L. texanus* as outgroup. Bayesian posterior probability values are presented above internodes. Bootstrap percentages recorded for maximum parsimony trees (1,000 replicates) and maximum likelihood full heuristic search (1,000 replicates) are shown below major branches in *bold* and *italics*, respectively. *Rectangles* highlight three exceptional individuals of the parasite which were out of *Eurypanopeus depressus* hosts, but group within the *Panopeus* clade. *Numbers* after site names depict individual parasites; identical haplotypes collapsed. A *scale bar* for the expected number of substitutions is given in the *lower left*

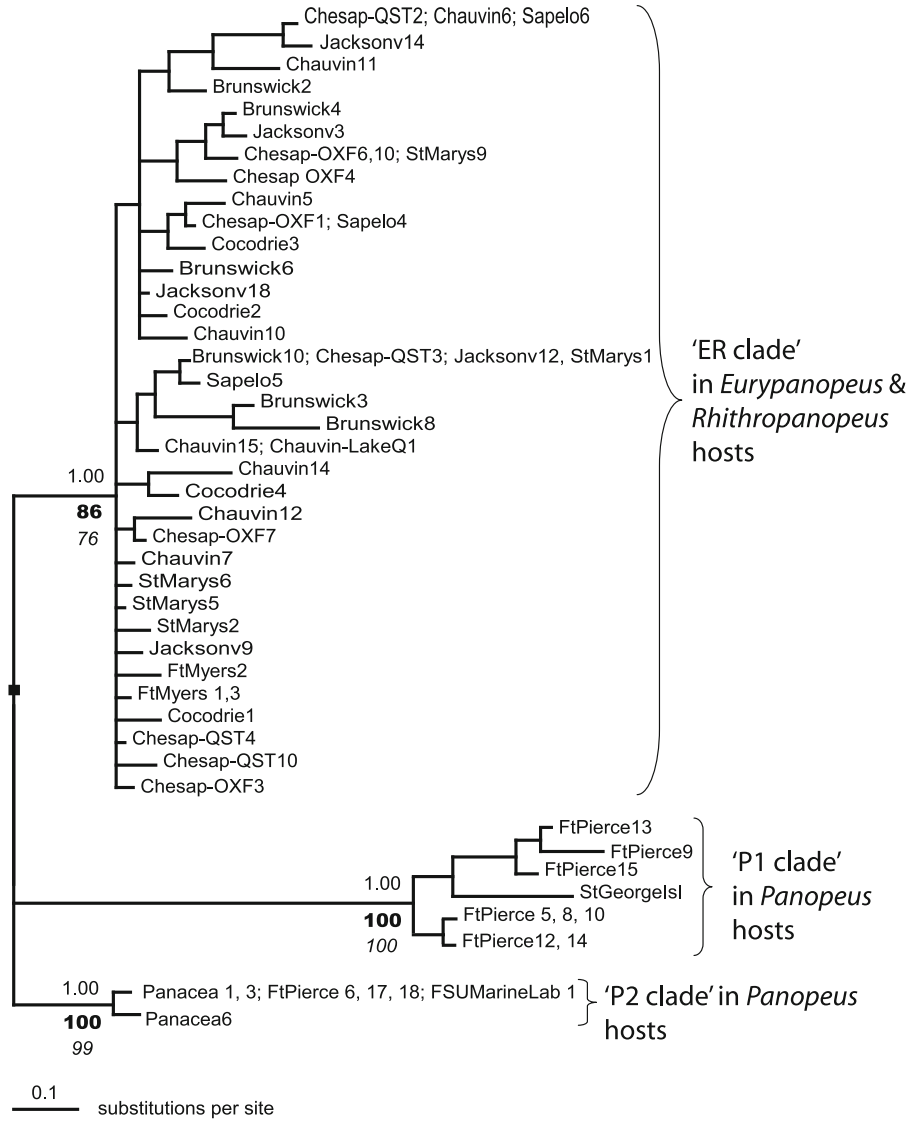

Fig. 4 Bayesian tree for *Loxothylacus panopaei* on the nuclear locus CytC. Bayesian posterior probability values are presented above internodes. Bootstrap percentages recorded for maximum parsimony trees (1,000 replicates) and maximum likelihood full heuristic search (100 replicates) are shown below major branches in *bold* and *italics*, respectively. *Numbers* after site names depict individual parasites; identical haplotypes collapsed. A *scale bar* for the expected number of substitutions is given in the *lower left*

Maximum likelihood (ML) and Bayesian analyses

Appropriate substitution models were selected by model testing, using the Akaike Information Criterion (AIC) per Posada and Buckley (2004) in MrModeltest V. 1.1b (Posada and Crandall 1998, 2001). AIC indicated that HKY + I (HKY = Hasegawa–Kishino–Yano model, I = invariable sites) represented the optimal model in respect to the COI dataset, while GTR + I + G (GTR = general time reversible model, I = invariable sites, G = gamma distributed rate variation among sites) was the optimal model for CytC.

ML analyses were performed using PAUP*, v4.0b10 (Swofford 2001) under the likelihood settings suggested from the model tests [for COI: Lset Base = (0.2985, 0.1348, 0.1475) Nst = 2 TRatio = 7.1716 Rates = equal Pinvar = 0.7429; for CytC:

Lset Base = (0.3928, 0.1585, 0.1472) Nst = 6 Rmat = (0.8737, 1.6898, 0.3813, 0.9083, 1.3823) Rates = gamma Shape = 0.6095 Pinvar = 0.4329]. For the COI locus, a branch and bound search was conducted to find the best tree and clade support was assessed with nonparametric bootstrap using 1,000 replicates, Tree Bisection Reconnection (TBR) branch swapping and 10 random sequence addition replicates. The COI tree was rooted to a consensus of four sequences of *L. texanus* as an outgroup. For the CytC locus, the best tree was searched under heuristic search option with TBR branch swapping and 100 random sequence addition replicates. Clade support was assessed with nonparametric bootstrap with 100 replicates.

Bayesian analyses were conducted using MrBayes 3.0B4 (Huelsenbeck and Ronquist 2001). For both datasets, two sets of four Markov chains in parallel, three heated and one cold, were started from a random tree and all 8 chains ran simultaneously for 1 million generations, with trees being sampled every 500 generations for a total of 2,000 trees. Convergence of chains was diagnosed using the deviation of split frequencies. After the likelihood of the trees of each chain converged as indicated by a split frequency below 5%, the first 800 trees (40%) were discarded as burn-in for the CytC dataset and the first 50 trees (2.5%) were discarded for the COI dataset. The majority-rule consensus tree containing the posterior probabilities of the phylogeny was determined from the remaining trees.

Maximum parsimony (MP) analysis

For the COI locus, an equally weighted maximum parsimony (MP) search was run by branch and bound. A bootstrap was conducted using 1,000 replicates, 10 separate heuristic searches, TBR branch swapping, holding one tree per step, and keeping all most-parsimonious trees. For the CytC locus, a heuristic MP search was run with TBR and repeated 100 times. Bootstrap support was calculated with 1,000 replicates and 10 separate heuristic searches and with TBR branch swapping.

Infection experiments

Experimental infections were conducted to test for host susceptibility to *L. panopaei*. Infected and uninfected crabs were collected between April 17 and May 24, 2006 (Table 2) and infection experiments, i.e. exposure of crabs to parasite larvae, were initiated from May 14 to July 27, 2006. Infected crabs from Edgewater, FL (the nonindigenous lineage of *E. depressus/R. depressus* infecting parasite) with full externae were cultured in 5 × 10 cm class jars, which were checked for nauplius larvae under the dissecting microscope every 2 days, when sea water was exchanged. Once nauplius larvae were present, they were filtered through 4 μm mesh and transferred into a new dish of fresh sea water where they were cultured to cyprid larval stage for infection trials.

Since only freshly molted crabs are vulnerable to an infection (Alvarez 1993), crabs from the Cape Canaveral region and from Fort Pierce were cultured until molting and then exposed within 24 h to cypris larvae of *L. panopaei* produced from infected crabs. Intermolt crabs destined for cypris exposure were cultured in groups of up to 15 crabs in 20 L aquaria filled with 5 cm of filtered aerated seawater by bubble stones until molting. Crabs from different source populations were maintained separately and were initially held in closed systems with water changed every 2 days. Later on, molting frequency of crabs was increased by using running sea water conditions. All crabs were provided with stones as shelter and were fed with pelleted food ('Crab & Lobster Bites', HBH Pet Products, Springville, UT, USA; 'Aquarian Sinking Shrimp Pellets for Bottom Feeders', Chalfont, PA, USA). All culturing was conducted at ambient Ft. Pierce temperatures (indoors, shaded conditions, no air-condition) and all sea water used had a salinity of approximately 35 ppt (parts per thousand; Fort Pierce Inlet sea water from the Smithsonian Marine Station's sea water supply).

Depending on how many crabs had molted within a 24 h period, 1–8 crabs were put into separate aquaria with fresh sea water in a closed system and combined with all *L. panopaei* cypris larvae from a single or up to three spawns. Aquarium sizes were chosen according to number of crabs (size range from 5 to 19 mm) and water level was maintained at 5 cm. This way, concentrations of parasite larvae varied according to aquarium size but were considered to be in excess compared to field conditions. Molted crabs that did not die or show virgin externae were held in culture for a minimum of 33 days after exposure to cypris larvae, which is the mean development time from infection to

emergence of the externa given by Alvarez et al. (1995). Those authors indicated a maximum time of externa emergence of 57 days, so we could have missed late-emerging infections.

Results

Parasite prevalence and distribution

A total of 4,011 xanthoid crabs of three species were sampled in 2006 from 11 locations along the Atlantic coast of Florida and 9 sites along GOM to determine prevalence (Table 2; Fig. 1). Visible externae indicated *L. panopaei* infection. In the introduced range (Atlantic coast north of Cape Canaveral), parasite populations were exclusively found in *Eurypanopeus depressus* hosts, 82 of 385 crabs were infected with prevalences ranging from 2 to 82% (21% on average) (Table 2). Over 200 crabs of the genus *Panopeus* examined from the same range were uninfected. In the Atlantic portion of the native range (south of Cape Canaveral), parasites were exclusively found in panopeid crabs; 42 of 222 crabs were infected with prevalences ranging from 0 to 100% (19% on average) whereas 460 syntopic *E. depressus* crabs were uninfected ('syntopic' used according to Rivas 1964 for *E. depressus* and panopaeid crabs living on the same oyster beds). Along Florida's west coast in the GOM prevalences were lower: 0–8% of panopeid crabs (5 of 336 crabs) and zero out of 240 *E. depressus* were infected in our samples (only Tolley et al. 2006 found 1% infected *E. depressus* at Ft. Myers by sampling thousands of crabs; three of these specimens are genotyped in this study). West of the Mississippi river, at Cocodrie in Louisiana, we found 1% infected *E. depressus* (4 of 447 crabs), but no *Rithropanopeus harrisii*. Very close to Cocodrie, at two sites in Chauvin, we found only *R. harrisii* and an average prevalence of 16% (19 of 117 crabs).

Mitochondrial and nuclear phylogeography

For the mitochondrial COI locus, 71 individual *L. panopaei* plus 4 individual *Loxothylacus texanus* (the outgroup) were genotyped for 438 basepairs. This locus resolved two clades of *L. panopaei* that were the same regardless of tree building method (clades ER and P; Fig. 3). Clade ER had high bootstrap supports (MP: 99 and ML: 90%) and a bayesian posterior probability of 0.95 and contained parasite individuals found exclusively in *E. depressus* and *R. harrisii* hosts. Hosts infected with this parasite lineage were in both the introduced and native (GOM) ranges (Figs. 1, 3). Clade P contained parasites exclusively from the native range, mostly in *Panopeus* hosts but also in three *E. depressus* individuals from the GOM (two from Cocodrie, Louisiana, and one from Ft. Myers, West Florida); clade P also had high bootstrap supports (MP: 100 and ML: 99%) and a bayesian posterior probability of 0.99. There were 11 haplotypes including 10 in clade ER (differing by pairwise sequence difference of 0.61% ± 0.36 SD) but only one haplotype in clade P from 20 specimens sequenced from *Panopeus* spp. hosts. The coding sequences between clades ER and P differed by 27 non-synonymous substitutions and a total of 7.5% sequence difference (average number of nucleotide substitutions per site).

The nuclear cytochrome c marker (CytC), where 398 basepairs from 61 individuals were sequenced and no outgroup exists, contained 77 parsimony-informative characters. This marker resolved three clades with identical topology using the three tree building methods (Fig. 4). Clade ER contained only parasites from *E. depressus* and *R. harrisii* hosts and was strictly concordant with the COI result (bootstraps ML: 76, MP: 86; bayesian posterior probability: 1.0). Two clades P1 and P2 were well-supported (bootstraps ML: 100 and 99, respectively, MP: both100; bayesian posterior probability: both 1.0) and contained only parasites infecting crab hosts of the genus *Panopeus.* Both nuclear and mitochondrial genetic loci showed that parasite lineages present in the Chesapeake Bay were also present in the GOM: sequences of *L. panopaei* from *E. depressus* and *R. harrisii* hosts of both loci from both regions fell into clades ER (Figs. 3, 4). Clade ER of the CytC locus contained 35 haplotypes (differing by pairwise sequence difference of 0.66% ± 0.40 SD), clade P1 6 haplotypes (1.29% ± 0.70 SD) and clade P2 2 (0.31% ± 0.22 SD) haplotypes. Comparison of the non-coding nucleotide differences between CytC clades shows 7 fixed nucleotide differences in clade ER versus clades P1 and P2. Average number of nucleotide substitutions per site for ER versus P1 was 8.3% and for ER versus P2 was 6.2%. There were 24 fixed differences and 2 indels in clade P1 versus clade P2 (10.2%).

Table 3 Experimental infection of molted *Eurypanopeus depressus* crabs by *Loxothylacus panopaei* larvae. Crabs were from parasite-free populations south of Cape Canaveral and parasite larvae were from the invasive parasite lineage north of Cape Canaveral. Emergence of a virgin externa indicated successful infection by female cypris larvae; 'no. of crabs dead' indicated molted crabs which died 2–8 days after combination with cypris larvae

Site	No. of crabs molted	No. of crabs dead	Virgin externa	Days before externa visible
Scottsmoore	4	1	1	38
Haulover Canal	9	2	0	
Titusville	4	2	1	32
406 Bridge	4	2	0	
405 Bridge	10	2	0	
Port Canaveral	5	1	3	42
				42
				34
Cocoa	2	1	0	
Fort Pierce	27	5	4	37
				32
				28
				31
Sum	65	16	9	
Average ± SD				35 ± 5

Infection experiment

A total of 65 newly molted *E. depressus* crabs, collected from parasite-free populations on Jack Island near Fort Pierce, were exposed to cypris larvae of *L. panopaei* obtained from *E. depressus* hosts collected in the invasive range of the parasite (Edgewater, FL, north of Cape Canaveral) (Table 3). These trials resulted in virgin externae emerging in 9 *E. depressus* (14%) trial hosts. The externae in these trials developed after a mean of 35 ± 5 days (mean ± SD). Of the 65 newly molted crabs, 16 (25%) died after combination with cypris larvae, which may or may not be due to lethal effects of the parasite (Thresher et al. 2000).

Discussion

Genetic diversity and host specificity

Two independent loci (mitochondrial COI and nuclear CytC) indicated that *L. panopaei* parasites infecting mud crab hosts of the genus *Panopeus* are genetically distinct from *L. panopaei* infecting *E. depressus* and *R. harrisii* hosts (Figs. 3, 4). For the nuclear marker this association between genetic lineage and host species was absolute. That is, host taxon appears to be 100% predictable from CytC genotype. Infection of *E. depressus* and *R. harrisii* hosts previously had been documented at low prevalence (1% and below) in Southeast and West Florida portions of the native range (Reinhard and Reischman 1958; Hines et al. 1997; Tolley et al. 2006), suggesting that these hosts might simply represent 'mistakes' or sub-optimal hosts for a primarily *Panopeus*-specific parasite. Our nuclear data indicate that these previous observations represent a genetically distinct *L. panopaei* lineage that exclusively infects *E. depressus* and *R. harrisii* hosts rather than indicating the existence of rare alternative hosts for a *Panopeus*-specific parasite.

However, based on the mitochondrial marker examined here three parasite individuals from *E. depressus* hosts fell into the '*Panopeus* host' clade, suggesting that they represent host range expansion in the direction of female parasites from *Panopeus* spp. hosts infecting *E. depressus* hosts. These patterns identify a completely unknown degree of parasite diversity but also raise several questions: (1) Are the mitochondrial 'cross-overs' indicative of persistent but dead-end cross-infections in mixed host populations, or remnants from historical introgression?

(2) How many parasite lineages are there, two or three, and what is their taxonomic status? (3) Which parasite lineage was introduced to Chesapeake Bay and from where? We address these questions in turn before addressing (4) the potential for constraints on further southward range expansion of the invasive parasite.

Cross-infection or historical introgression?

Our genetic data provide some insights into the potential for interbreeding between 'ER' and 'P' clade parasites because when sampling mature externa, female tissue plus fertilized eggs was collected. In the case of a hypothetical cross-infection from crabs of the genus *Panopeus* to an *E. depressus* host, the genotypic signature should be clade P for the mtDNA. The parasite's nuclear genome will be heterozygous for clade ER and clade P sequences if male cyprid larvae use the host crab as a cue for finding virgin externae of receptive females (ER male cyprid larvae most likely to fertilize), or genotypes would be homozygous for P clade sequences if male cyprid larvae use cues from the parasite itself (P clade male cyprid larvae will find and fertiize a P clade female regardless of host). The three cross-over individuals did not have CytC heterozygosity attributable to sequences from different clades. Therefore, our results generate two testable hypotheses about interbreeding between the ER and P clades. One possibility is that cross-infection is ongoing at low rates but never leads to nuclear hybridization because *L. panopeai* male cyprid larvae ignore host and discern parasite females from the two clades. The other hypothesis is that cross-infection is rare, but occurred historically in the direction of *Panopeus* to *E. depressus* hosts, and resulted in mtDNA introgression (and "capture") while the proportion of nuclear genome deriving from the P clade parasite waned over subsequent back crosses. This hypothesis describes a pattern more often found in plant plastids (e.g. Rieseberg et al. 1990) and implies that there is no selection against the foreign mtDNA. We are unable to reject either of these hypotheses at present.

Taxonomic status of parasite lineages

With the potential for interbreeding uncertain based on existing data (as discussed above), we remain agnostic with respect to whether the three lineages resolved by the nuclear marker are distinct species according to the biological species concept. Nonetheless, nearly complete reproductive isolation is likely given: (1) the large divergence between clades; and (2) the fact that CytC, a nuclear locus capable of recombination, shows a well supported ER clade even after including Gulf of Mexico (GOM) specimens collected where interbreeding with P clade parasites is possible (e.g., Ft. Myers).

In the CytC tree, two distinct '*Panopeus* host' clades (clade P1 & clade P2, see Fig. 4) of *L. panopaei* were resolved and had a deeper divergence than their common ancestor had to the ER clade. Interestingly, these two lineages are sympatric in Southeast Florida. This fact, coupled with the high genetic divergence between P1 and P2 clades, suggests that they represent reproductively isolated species (see Fig. 4). However, this conclusion is at odds with the mtDNA phylogeny which shows no hint of this distinction, yet is expected to provide a more sensitive record of cladogenesis (Moore 1995; Palumbi et al. 2001). To the contrary, no haplotype variation was found among *L. panopeai* infecting *Panopeus* spp. hosts. The simplest hypothesis to explain this discordance is that recent mitochondrial DNA introgression between P1 and P2 populations led to a sweep and fixation of a single advantageous haplotype. However, with only two loci sampled, it remains to be seen which locus is giving a more accurate representation of population differences.

The evolutionary divergence between clades P1 and P2 may indicate greater host specificity of *L. panopaei* lineages within the crabs of the genus host species complex. There are at least five *Panopeus* species along the distributional range of *L. panopaei*: *P. lacustris, P. herbstii* (treated here as =*P. simpsoni*), *P. obesus, P. americanus* and *P. occidentalis*. Similar morphology among these species renders their classification and distinction difficult (Schubart et al. 2000). For example, questions remain as to whether *P. simpsoni* should be placed back into synonymy with *P. herbstii* and there is also a discrepancy between results of Hines et al. (1997), who very rarely found *P. herbstii* south of Cape Canaveral and no *L. panopaei* infections in this species, and the present study where crabs infected with *L. panopaei* in the Indian River Lagoon in Southeast Florida were identified as "*P. herbstii* (=*P. simpsoni*)" (D. L. Felder, personal communication). New molecular markers

appear to distinguish populations of *Panopeus* species listed above, though these findings have yet to be published (D. L. Felder, personal communication). Thus, for future studies on *L. panopaei* analysis of diagnostic mtDNA and nuclear DNA sequence characters of *Panopeus* hosts may prove informative.

We have limited data on taxonomy of infected panopeid crabs to correlate with the CytC clade structure of their parasites. From ethanol preserved crab samples a total of 8 Ft. Pierce and GOM crabs from clades P1 and P2 were identified to species by morphological characters: 5 out of 9 specimens in the *Panopeus* clade P1 were identified as *Panopeus herbstii* and one as *P. lacustris*. From *Panopeus* infecting clade P2, 2 out of 7 specimens were identified as *P. obesus* and one as *P. occidentalis* (D. L. Felder, personal communication). None of these identifications contradict the hypothesis of host-specific *L. panopaei* clades within the genus of *Panopeus* hosts.

We are aware of only a single reference, in which *L. panopaei* specimens from different hosts were compared morphologically. Reinhard and Reischman (1958; Figs. 2, 3, 4 therein) found differences in minute hair-like excrescences or spines in the external cuticle of the mantle: These spines measured 6–20 microns on parasites of *E. depressus* and *R. harrisii*, but attained a length of 44 microns on the parasite of *Panopeus herbstii*. On another specimen from *P. herbstii* from Louisiana, a third shape of these spines was found. These had rounded tips, instead of tips divided into 2 or 3 points as found in the two other shapes. Considering that after the publication of Reinhard and Reischman (1958) *P. herbstii* was split up into six species (Williams 1983), these three morphological spine types may correlate with different host spectra and possibly with our three CytC clades, which remains to be tested. We recommend that further studies analyze the above mentioned morphological characters in population samples in respect to the identification of specific host taxa.

Newly discovered genetic lineages in parasites have overturned traditional notions about ecological specialization in interspecific interactions (e.g. Bickford et al. 2007). With their more limited host spectra, the two (or three) *L. panopaei* lineages, which may represent two (or three) species, are also more specialized than thought previously, for example by Hines et al. (1997) to whom host specificity of *L. panopaei* appeared to be relatively generalized in comparison to other rhizocephalans. Considering the distinct geographical distributions of the clades P and ER, it may be that these two lineages or species also have narrower niches with respect to their abiotic environmental tolerances.

Which lineage was introduced to Chesapeake Bay and from where?

Our results suggest that three lineages of *L. panopaei* with different preferred hosts may occur in the GOM, and only one of these appears to have invaded Chesapeake Bay: Chesapeake Bay haplotypes, which were all from *E. depressus* and *R. harrisii* hosts, grouped only in the same clades resolved from both loci as GOM parasites from the same hosts.

Our results appear to reject a previous hypothesis that the parasite's preferred hosts changed after invasion, i.e., that host range expansion had occurred (Kruse and Hare 2007). Thus, flexibility in choice of different host species might not have contributed to invasion success. However, Hines et al. (1997) recorded a single incidence of parasitism of *Neopanope sayi* in Chesapeake Bay, which was a new host species that had not been recorded elsewhere. Thus it is possible that in the context of the invasion the parasite infected new hosts, but prevalence in new hosts has not increased beyond rare cases.

New records of *L. panopaei* in Brazil in the area of Pernambuco (Farrapeira et al. 2008; Farrapeira 2010) mention *P. occidentalis* and the grapsid crab *Aratus pisonii* as host species. However, before this can be considered a new invasion of *L. panopaei* with host range expansion, it is necessary to explore if this parasite is not cryptogenic, preferably with the genetic markers used in this study. Species are called cryptogenic if there is no definite evidence of their native or introduced status (Carlton 1996).

For the invasion of *L. panopaei* into Chesapeake Bay, we support and specify the following scenario first invoked by Van Engel et al. (1966): (1) *E. depressus* and/or *R. harrisii* in the GOM were infected with the *L. panopaei* ER clade (2) *L. panopaei* were transported from the GOM to the Chesapeake Bay in *E. depressus* and/or *R. harrisii* associated with oysters, and (3) the same native crab host species were subsequently infected by this parasite in Chesapeake Bay. Based on our results of higher prevalences west of the Mississippi river (native range; 4% averaged

over 3 sites and up to 16%) compared to very rare infections along the coasts of the states of Mississippi, Alabama and West and Southeast Florida, we infer that the ER clade *L. panopaei* introduced into Chesapeake Bay possibly orginated in the western GOM.

Interestingly, no matter which population of the *E. depressus*- and *R. harrisii*-specific parasite is regarded as the source for the introduction, prevalence of infection for *L. panopaei* in *E. depressus* that invaded the Cheasapeake Bay and spread southward, were always much higher (21% averaged over 3 sites and up to 82%) than it is in the same crab species in the GOM (<1% and up to 16%). Comparison with data of the invaded range from earlier studies reveals an even stronger difference (Hines et al. 1997: epidemic outbreaks and prevalences from 5 to 91% indicating high spatio-temporal variation; Kruse and Hare 2007: prevalences always at least 30% and up to 63%, in appropriate sample sizes). Also, Hines et al. (1997) highlighted that the native range of parasites in the Indian River Lagoon (below Cape Canaveral) exhibited consistently low prevalences, and epidemic prevalences were never observed. This may suggest higher infectivity of the invading parasite and/or higher susceptibility of naive host populations in the invaded range because of lack of co-evolution with that parasite as suspected earlier by Kruse and Hare (2007).

Prediction of range expansion across a biogeographic boundary

Compared to data from 2004 to 2005 (Kruse and Hare 2007), the leading front of the invasive lineage of *L. panopaei* in 2006 was located 50 km further south, in Titusville, Florida (Table 2; Fig. 2). This means that the invasive and indigenous populations of *panopaei* were located only about 40 km apart. We predict that the southward range expansion of nonindigenous *L. panopaei* has the potential to continue based on our results and several physical factors. First, our laboratory infection experiments showed that *E. depressus* from Southeast Florida in the vicinity of Fort Pierce and Cape Canaveral (Fig. 1) are not immune to *L. panopaei* collected from the northern invasive population at Edgewater. Second, our data showed a continual southward range expansion of invasive *L. panopaei* beyond the only obvious potential barrier to dispersal along the intracoastal waterway, the narrow Haulover Canal. Thus, this parasite has no problem dispersing through the tidal lagoons along east Florida. Third, there does not appear to be any competitive barrier to the nonindigenous parasite moving south beyond Titusville, because it infects different host species compared to the native *L. panopaei* found south of Cape Canaveral.

The geography of invasion for many species seem to agree with 'climate matching' expectations in which climatic features of a species' native range distribution predict the potential for successful establishment if transported to other regions (Vermeij 1991; Prinzing et al. 2002). Within the GOM, we identified the temperate northwestern region to be a possible source region for the Chesapeake Bay invasion of *L. panopaei*. If so, then warm subtropical temperatures in the southern Indian River may still limit the southward invasion of *L. panopaei*. Winter isotherms (Fig. 2) illustrate the temperature gradient that probably explains much of the observed transition from temperate to subtropical marine communities (Vermeij 1978). The existence of rare *E. depressus* infections in Ft. Myers and Fort Pierce (Hines et al. 1997; Tolley et al. 2006), relative to that found in Mississippi, might be an indication that conditions for this *L. panopaei* lineage are poor in Southeast Florida. In case further southward spread is not realized in the next years, high temperatures become more likely to impede spread of *L. panopaei,* as our infection experiments exclude physiological incompatibility of *E. depressus* hosts in Southeast Florida. We view this natural experiment as a valuable opportunity to evaluate the southern range restrictions on this invasive parasite.

Acknowledgments We wish to thank S. Reed, T. and E.-G. Kruse, D. Beissel, S. Altman, P. Rodgers and J. O'Brien for help on field trips and the rest of the staff at SMS at Fort Pierce for general support. Specimens of *L. panopaei* from Fort Myers were kindly provided by G. Tolley. We are grateful to D. Felder for species identifications of panopaeid crabs and to W. Herrnkind for sampling advice for the Florida panhandle. Christoph Bleidorn, associate editor Carol A. Stepien and three anonymous reviewers provided valuable comments to drafts of the manuscript. This work was supported by a 'Postdoctoral Fellowship at the Smithsonian Marine Station at Fort Pierce' from the Smithsonian Institution to I. K. and by Smithsonian Marine Science Network funds to A. H. H.

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