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Diversity and distribution of *Perkinsus* spp. along the coast of China: Implications for widespread transmission of *Perkinsus* spp. in mollusks

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Perkinsus species, which are parasitic pathogens of mollusks, have been transmitted and dispersed to various molluscan species along the coastal waters of many countries. However, few studies have addressed the diversity and distribution of *Perkinsus* spp. along the coast of China. Here we used conventional PCR amplification and sequencing techniques, combined with a qPCR assay as a confirmatory test, to evaluate the prevalence of *Perkinsus* species in molluscan species among different sea regions of China. Three *Perkinsus* species—*P. olseni*, *P. beihaiensis*, and *P. chesapeaki*—were detected, with *P. chesapeaki* reported for the first time along the Chinese coast. Seven of eight molluscan species carried *Perkinsus* species, including *Crassostrea gigas*, *C. hongkongensis*, *Sinonovacula constricta*, *Ruditapes philippinarum*, *Scapharca subcrenata*, *Meretrix lyrate*, and *Haliotis diversicolor*. *Perkinsus olseni* was prominent in the Yellow and Bohai Sea and East China Sea, while *P. beihaiensis* was prominent in the South China Sea. Most of the molluscan species carried *Perkinsus* spp. with the medium or low levels of PCR-prevalence (<30%). The three *Perkinsus* species possess high levels of internal transcriber spacer haplotypes, some of which are shared among many countries. The much higher PCR-prevalence of *Perkinsus* spp. in the clam *Ruditapes philippinarum* and the oyster *Crassostrea hongkongensis* suggests that *Perkinsus* species may be transmitted and dispersed to other mollusks through the transportation of *Perkinsus*-carried *R. philippinarum* and *C. hongkongensis*. *Perkinsus* carrying tended to be generally linked with a broader geographic range, lower prevalence, more diversified molluscan hosts, and more diversified *Perkinsus* haplotypes.

KEYWORDS

***Perkinsus* spp. distribution in China Perkinsus, mollusks, diversity, distribution, *Perkinsus chesapeaki*, Chinese coast**

Introduction

Mollusks are popular seafoods and account for over 80% of marine aquaculture production, making them the most cultured species in global mariculture in recent decades (Yang, 2016; FAO, 2020). China has the largest marine molluscan aquaculture areas, the largest number of cultivated molluscan species, and the highest molluscan production in the world (Bureau of Fisheries of the China's Ministry of Agriculture and Rural Affairs, 2020a; FAO, 2020). However, molluscan diseases occur frequently in China, and the molluscan industry has sustained severe economic losses in recent years. In 2019, the economic loss of mollusks owing to disease reached 9.51 billion RMB, accounting for 23.3% of the total economic loss of aquaculture (Bureau of Fisheries of the China's Ministry of Agriculture and Rural Affairs, 2020b).

As potentially devastating pathogens, the protozoan parasites of the genus *Perkinsus* have caused large-scale mortality in many molluscan species (e.g., oysters, abalones, clams, scallops, pearl oysters, cockles, mussels) worldwide, resulting in large economic losses (Villalba et al., 2004; Choi and Park, 2010; Dungan and Reece, 2020). Thereinto, *P. marinus* and *P. olseni*, have been described as internationally reportable or notifiable pathogens for mollusks on the list of the World Organization for Animal Health (OIE, https://www.oie.int/en/produit/manual-of-diagnostic-tests-for-aquatic-animals-2021/). Increasing numbers of molluscan species have been found to be infected by *Perkinsus* spp., including some wild molluscan species (Brousseau et al., 1998; Cui et al., 2018; Dungan and Reece, 2020), which suggests that *Perkinsus* spp. are widely distributed and have been transmitted to most molluscan species. *Perkinsus* spp. tend to be more geographically widespread and diverse, and an increasing number of countries and regions have detected infection by several species of *Perkinsus* (Neto et al., 2016; Pagenkopp Lohan et al., 2016; Shamal et al., 2018).

As a result of various ecological, epidemiological, and evolutionary determinants, parasite species biodiversity exhibits a range of geographic patterns (Poulin et al., 2011; Morand, 2015). Moreover, host traits and parasite specificity may shape parasite biogeography (Krasnov et al., 2008; Poulin et al., 2011). Generally, parasite species richness is assumed to be high in the tropics, matching the high host diversity there (Torchin et al., 2015). China has 18,000 km of continental coastline in the western Pacific that crosses approximately 50 degrees of latitude from the south to north and includes three distinctly different sea regions (the Yellow and Bohai Sea, East China Sea, and South China Sea). China has more than 800 molluscan species, approximately 20 of which had been farmed commercially on a large scale along the shoreline. Whether the distribution and diversity of *Perkinsus* spp. have been affected by latitudinal gradients and host traits is relatively unknown and has yet to be monitored in the sea regions of China.

In this study, we used conventional PCR amplification and sequencing techniques, combined with a qPCR assay as a confirmatory test, to evaluate the prevalence of *Perkinsus* spp. in molluscan species among different sea regions of China. The internal transcriber spacer (ITS) sequences obtained here were combined with related *Perkinsus* spp. ITS sequences in NCBI Genbank and used to analyze the global haplotypes and their distribution in China. Our study mainly focuses on three areas: 1) the species diversity of *Perkinsus* spp. in different sea regions of China; 2) the geographical distribution of *Perkinsus* spp. in China; and 3) the diversity and distribution of *Perkinsus* spp. haplotypes. Our study provides a comprehensive assessment of *Perkinsus* spp. distribution and evaluates the ranges of molluscan species carried *Perkinsus* spp., thus providing a solid foundation for the prevention and control of perkinsosis in mollusks in China.

Materials and methods

Sampling

From August to September of 2017, as many molluscan species as possible were collected from various intertidal and subtidal habitats, or purchased from local farmers who collected them along local coastal waters, at multiple locations in three contiguous geographical regions of China, namely the Yellow and Bohai Sea (herein YBS), East China Sea (ECS), and South China Sea (SCS). Following the measurement of shell length, the gill, mantle, and digestive gland tissues of each live molluscan specimen were dissected using forceps, excised, and preserved in 95% (v/v) ethanol for further molecular assays. To avoid extracting DNA from the filtered particles attached onto the gill surface, gill tissue from each sample was completely washed *via* sterile Pasteur pipette at least three times with 0.2 M phosphate-buffered saline (PBS, pH 7.4) before being preserved in 95% (v/v) ethanol. For the convenience of *Perkinsus* spp. carrying comparisons, we selected as target samples the molluscan species that most frequently occurred at multiple locations of each typical sea region. Detailed information on molluscan sampling is shown in Table 1. Briefly, three locations at each sea region were screened as target locations, namely Dalian (DL), Qinhuangdao (QH), and Rizhao (RZ) located in the YBS; Taizhou (TZ), Ningde (ND), and Putian (PT) in the ECS; and Shantou (ST), Yangjiang (YJ), and Beihai (BH) in the SCS (Figure 1). To detect the carrying of *Perkinsus* species, a total of 1,410 individuals representing eight molluscan species were used, including *Crassostrea gigas*, *C. hongkongensis*, *Sinonovacula constricta*, *Ruditapes*

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TABLE 1 Sampling information of molluscs along the coastal waters of China.

Region	Location	Molluscan species	N	Shell Length (mm)
Yellow and Bohai sea (YBS)	Dalian (DL)	*C. gigas*	30	96.72 ± 10.06
		Si. constricta	30	61.66 ± 3.54
		R. philippinarum	30	39.81 ± 2.10
		Sc. subcrenata	30	43.02 ± 2.12
		M. lyrata	30	47.93 ± 1.83
		H. discus hannai	30	63.58 ± 1.99
	Qinhuangdao (QH)	*C. gigas*	30	79.73 ± 10.94
		Si. constricta	30	54.33 ± 3.78
		R. philippinarum	30	37.89 ± 1.24
		Sc. subcrenata	30	40.11 ± 2.72
		H. discus hannai	30	62.33 ± 2.39
	Rizhao (RZ)	*C. gigas*	30	84.81 ± 8.29
		Si. constricta	30	50.83 ± 4.12
		R. philippinarum	30	34.86 ± 3.47
		Sc. subcrenata	30	41.66 ± 3.10
		M. lyrata	30	45.21 ± 3.31
		H. discus hannai	30	54.42 ± 1.68
East China Sea (ECS)	Taizhou (TZ)	*C. gigas*	30	115.87 ± 14.21
		Si. constricta	30	64.31 ± 3.62
		R. philippinarum	30	36.86 ± 2.49
		Sc. subcrenata	30	30.37 ± 2.23
		M. lyrata	30	49.76 ± 2.46
	Ningde (ND)	*C. gigas*	30	86.14 ± 8.69
		Si. constricta	30	69.64 ± 3.43
		R. philippinarum	30	35.89 ± 2.53
		Sc. subcrenata	30	26.77 ± 2.16
		H. diversicolor	30	54.64 ± 2.10
	Putian (PT)	*C. gigas*	30	43.05 ± 4.85
		Si. constricta	30	49.90 ± 2.57
		R. philippinarum	30	37.09 ± 1.49
		Sc. subcrenata	30	40.44 ± 4.15
		H. diversicolor	30	69.79 ± 3.66
South China Sea (SCS)	Shantou (ST)	*C. hongkongensis*	30	87.35 ± 8.57
		Si. constricta	30	51.44 ± 3.14
		R. philippinarum	30	35.63 ± 2.13
		Sc. subcrenata	30	27.24 ± 1.27
		M. lyrata	30	41.19 ± 2.42
		H. diversicolor	30	67.38 ± 2.60
	Yangjiang (YJ)	*C. hongkongensis*	30	93.93 ± 11.61
		Si. constricta	30	56.08 ± 3.13
		R. philippinarum	30	44.84 ± 2.23
		M. lyrata	30	43.51 ± 2.52
	Beihai	*C. hongkongensis*	30	72.55 ± 5.18
		Si. constricta	30	69.27 ± 5.44
		R. philippinarum	30	41.94 ± 4.14
		Sc. subcrenata	30	36.52 ± 2.56
		M. lyrata	30	41.44 ± 2.57
		Total number	1410	

C, Crassostrea; Si, Sinonovacula; R, Ruditapes; Sc, Scapharca; M, Meretrix lyrata; H, Haliotis.

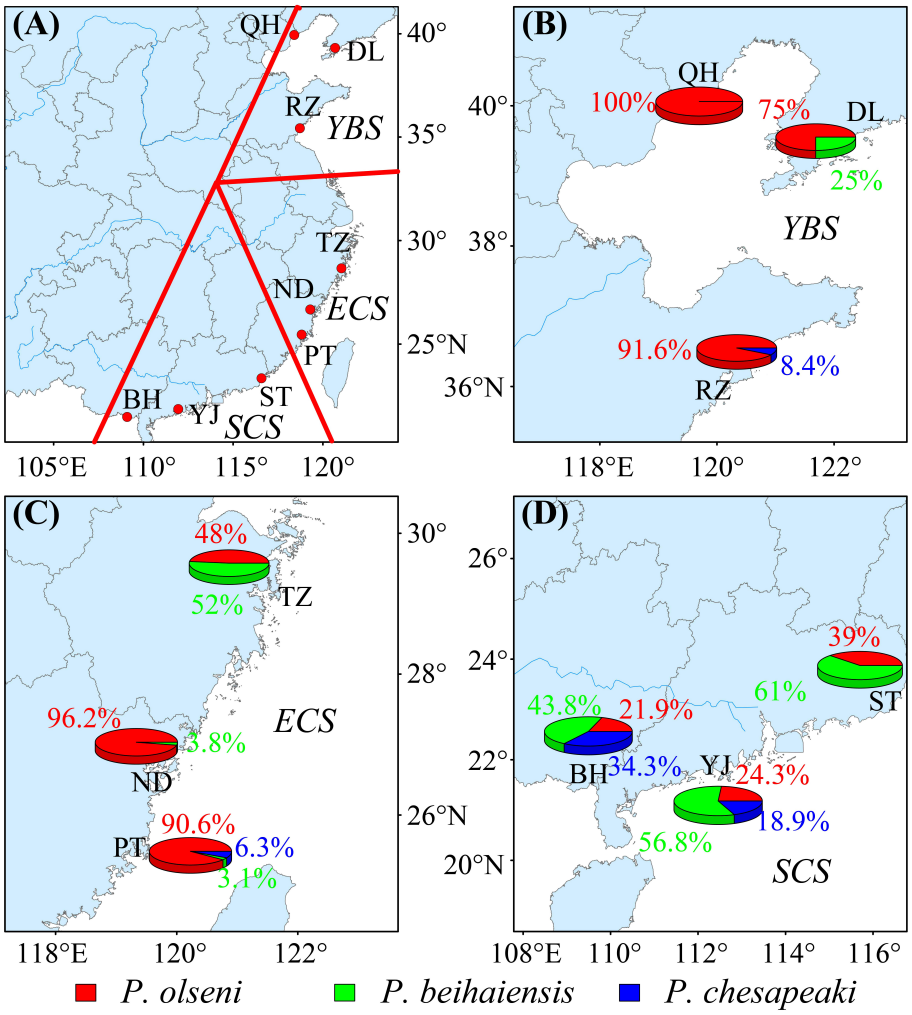

FIGURE 1
Map of sampling locations along the coast of China **(A)** and *Perkinsus* species carrying rate in molluscan species at different locations in the Yellow and Bohai Sea (YBS) **(B)**, East China Sea (ECS) **(C)**, and South China Sea (SCS) **(D)**. DL, Dalian; QH, Qinghuangdao; RZ, Rizhao; TZ, Taizhou; ND, Ningde; PT, Putian; ST, Shantou; YJ, Yangjang; BH, Beihai.

philippinarum, *Scapharca subcrenata*, *Meretrix lyrate*, *Haliotis discus hannai*, and *H. diversicolor*. At each location, 30 individuals of each molluscan species were randomly sampled (Table 1).

DNA extraction, PCR amplification, and sequencing

Approximately 100 mg of gill, mantle and digestive gland tissue was cut individually, and mixed in a 1.5-ml Eppendorf tube. The three tissues of each sample were then homogenized in 0.1 M sodium phosphate buffer. The resulting tissue homogenate was subjected to DNA extraction procedures using MicroElute Genomic DNA Kit (Omega, USA) according to manufacturer's instructions.

The genus-specific primer pairs, PerkITS85 (5′ CCGCTTTGTTTGGMTCCC3′) and PerkITS750 (5′ ACATCAGGCCTTCTAATGATG3′) (Casas et al., 2002), were used to amplify an approximately 700-bp fragment of the ITS region of *Perkinsus* spp. PCR was performed in a 25-μL volume consisting of 12.5 μL of 2×PCR Mix (Dongsheng Biotech, Guangzhou, China), 1 μL each of forward and reverse primers (10 μM), 1 μL of template DNA, and 9.5 μL of ultrapure water. A positive control (1,000 copies of *P. beihaiensis*-pMD 18T recombinant plasmid DNA) and a negative control (nuclease-free water) were included in each PCR run. The amplification was performed with an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C

for 1 min, with a final elongation at 72°C for 7 min. The PCR results were confirmed on 1% agarose gel stained with SYBR green. Samples were considered positive PCR products if a product of ~700 bp was detected, the positive control showed the same band size, and the negative and nontemplate controls showed no bands. Positive PCR products were sequenced in both directions using amplification primers and ABI Big Dye Terminator Chemistry on an ABI 3730XL automatic DNA sequencer (Applied Biosystems, USA).

The forward and reverse sequences from the same sample were assembled to one consensus sequence using SeqMan 4.0 (DNAStar, Madison, WI, USA) and manually edited to ensure that all bases within the consensus sequence were identical in both the forward and reverse sequences. The species of *Perkinsus* present in each molluscan sample were determined by comparing the target sequences with those in GenBank. When the target sequence of the species showed 99.5%–100% identity to that of *Perkinsus* spp. deposited in GenBank, the identified species was regarded as *Perkinsus* spp. (Cui et al., 2018).

qPCR assay for confirmation

According to the sequencing results, 349 positive samples were identified as three *Perkinsus* species, namely *P. olseni*, *P. beihaiensis*, and *P. chesapeaki*. A confirmatory qPCR test was performed among the 349 positive samples to minimize the number of false positive samples. For each sample, *Perkinsus*-specific qPCR assays were run for *P. olseni* (POF: 5′-GAG TGTCTC TGGTTGCTCGCA A-3′; PO-R: 5′-ACA TCAGGC CTT CTA ATG ATG-3′) (Cui et al., 2018), *P. beihaiensis* (Q2-F: 5′-TCGATGAAGGACGCAACGAA-3′; Q2-R: 5′-CTCATTTCTGCGGGGCTACA-3′) (Wu et al., 2019), and *P. chesapeaki* (forward primer 5′-AAG TCG AAT TGG AGG CGT GGT GAC-3′; reverse primer 5′-ATT GTG TAA CCA CCC CAG GC-3′) (Marquis et al., 2020). The sets of primers for *P. olseni* and *P. beihaiensis* target the ITS region, and the set of primers for *P. chesapeaki* targets the NTS (non-transcribed spacer) region. DNA standards for qPCR were prepared from qPCR-positive samples that were cloned and serially diluted 10-fold over six orders of magnitude (2.6×10^7 to 2.6×10^2 copies/μL). The qPCR was carried out in a 10 μL reaction, comprising 5 μL TB Green™ Premix (TaKaRa, Kusatsu, Japan), 0.5 μL of each primer (10 μM), 1 μL of extracted DNA and 3 μL of nuclease-free water. Reactions were conducted in triplicate on an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) using the Eco System Software with the following temperature program: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 40 s. In all reactions *P. olseni*, *P. beihaiensis*, or *P. chesapeaki* DNA was used as a positive control, and DNase-free water as the non-template control. A sample was considered positive if the qPCR threshold cycle (CT) value of the product rose above zero before the 30th cycle, at the same time, the qPCR CT value of the negative and non-template controls were zero before the 30th cycle.

According to the confirmatory qPCR test results, of the 349 conventional PCR-positive samples, 306 samples tested positive and 43 samples negative. Therefore, 306 molluscan samples that tested positive in both the conventional PCR test and qPCR test were deemed carrying the *Perkinsus* spp.

Diagnosis of *Perkinsus* spp. carrying

The PCR-prevalence of *Perkinsus* spp. was considered to be the percentage of infected mollusks in the total samples. The PCR-prevalence of *Perkinsus* spp. was divided into four PCR-prevalence grades: 1) no carrying (PCR-prevalence 0%); 2) low (PCR-prevalence greater than 0 and less than or equal to 20%); 3) medium (PCR-prevalence greater than 20% and less than or equal to 50%); and 4) high (PCR-prevalence greater than 50%). The carrying rate of *Perkinsus* spp. was defined as the ratio between the number of mollusks carrying the specific *Perkinsus* sp. and the total number of *Perkinsus*-carried mollusks in the specific sampling location.

Haplotype analysis

To generate global haplotype networks for each species, all sequences of the specific *Perkinsus* species in the present study were combined with the same *Perkinsus* species sequences obtained from GenBank. Only those sequences that definitely annotated the source country information in GenBank were used. For each *Perkinsus* sp., sequences were aligned using Clustal X 1.83 (Thompson et al., 1997) with the default parameters. The resulting alignments were manually edited using the program BioEdit (Hall, 1999). To obtain the equal lengths of all aligned sequences, alignments were trimmed at both ends. In the resulting alignments, the sequences that included ambiguous bases (e.g., degenerate bases) were deleted to avoid an inflation of haplotypes.

Haplotype networks were preliminarily generated with these alignments using the haplotypes package (Aktas, 2020) in the R software environment. For each *Perkinsus* sp. preliminary haplotype network, if single haplotypes included multiple sequences from the same country, then only the unique sequences were retained in that alignment. The sequences that had unique haplotypes in China but no sequence records in GenBank were submitted to NCBI and registered in GenBank (accession nos. MT908881–MT908909). Following these sequence screenings, three alignments were ultimately used to create the global networks, including a 481-bp alignment containing 171 sequences for *P. beihaiensis*, a 497-bp alignment containing 378 sequences for *P. olseni*, and a 492-bp alignment containing 86 sequences for *P. chesapeaki*.

Haplotype networks were generated with those screened alignments using the haplotypes package (Aktas, 2020) with its default parameters in the R software environment. Each haplotype corresponded to the country from which it was sampled in the haplotype network analyses. The haplotype networks that were generated were exported using the export package (Wenseleers and Vanderaa, 2018) to Microsoft PowerPoint and slightly revised to a final graph version.

Statistical analysis

Statistical analyses were performed in the R software environment. Pairwise comparisons of *Perkinsus* spp. PCR-prevalence among different regions and molluscan species were conducted using the Chi-square test in the stats package (R Core Team, 2020) with the default parameters. Statistical significance was set at $P < 0.05$. Except for the haplotype networks graphs, all graphs were drawn using the ggplot2 package (Wickham, 2016) in R.

Results

Species diversity of *Perkinsus* spp.

Three *Perkinsus* species—*P. olseni*, *P. beihaiensis*, and *P. chesapeaki*—were detected in molluscan species along the coast of China's waters (Figure 1). The carrying rates of *P. olseni* ranged from 75% to 100% at the sites located in the YBS and from 48% to 96.2% in the ECS sites (Figure 1), which were clearly higher than those in the SCS (21.9%–39%). The carrying rates of *P. beihaiensis* ranged from 43.8% to 61% at the sites located in the SCS, which were higher than those in the ECS (3.1%–52%) and YBS (0%–25%) (Figure 1). The carrying rates of *P. chesapeaki* were far less than those of *P. olseni* and *P. beihaiensis*, and *P. chesapeaki* was mainly carried by the molluscan species located in the SCS (Figure 1).

The PCR-prevalence of *P. olseni* was highest in *R. philippinarum* (51.8%) and clearly higher than that in other molluscan species (Figure 2). The highest PCR-prevalence of *P. beihaiensis* occurred in *C. hongkongensis* and was also evidently higher than that in other molluscan species (Figure 2). The PCR-prevalence of *P. chesapeaki* was at the low carrying grade in molluscan species, and the highest PCR-prevalence was in *S. subcrenata* (only 4.2%).

Seven of eight molluscan species carried *Perkinsus* species, including *Crassostrea gigas*, *C. hongkongensis*, *Sinonovacula constricta*, *Ruditapes philippinarum*, *Scapharca subcrenata*, *Meretrix lyrate*, and *Haliotis diversicolor* (Figure 3).

Geographical distribution of *Perkinsus* spp.

The PCR-prevalence of *Perkinsus* spp. at most locations were at the medium carrying grade and did not differ significantly (Figure 3). The PCR-prevalence of *Perkinsus* spp. ranged from 15.3% (in BH) to 33.3% (in TZ). The PCR-prevalence of *Perkinsus* spp. was higher in the SCS (24.2%) than in the ECS (22.4%) and YBS (18.8%) (Figure 3; Table 2).

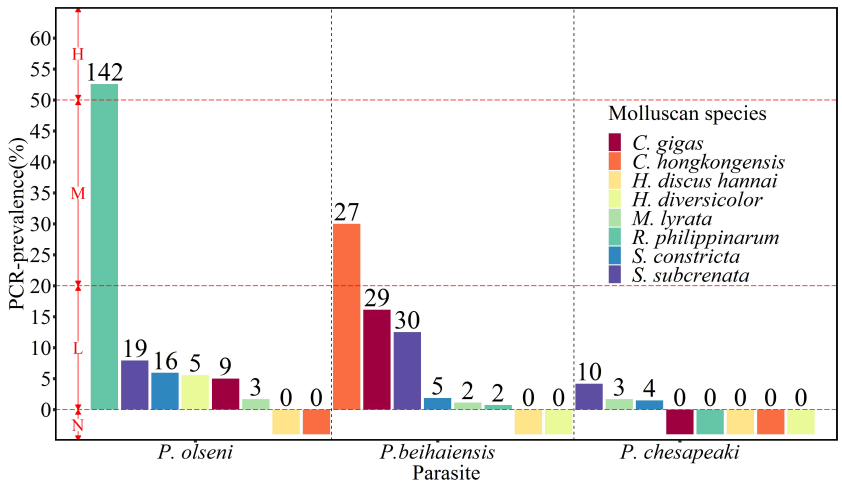

FIGURE 2
Perkinsus species diversity in different molluscan species in waters along the coast of China. Numbers above the bars indicate the total number of *Perkinsus*-carried individuals for each molluscan species. H: high PCR-prevalence grade; M: medium PCR-prevalence grade; L: low PCR-prevalence grade.

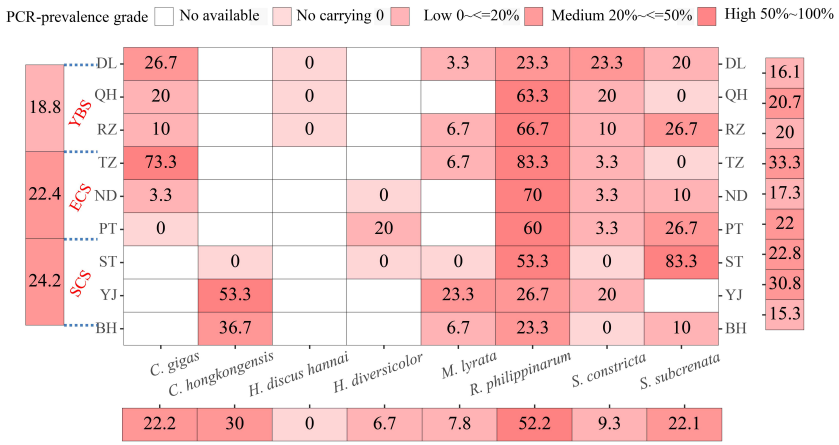

FIGURE 3
Heat plot revealing PCR-prevalence of *Perkinsus* species in different molluscan species, locations, and sea regions along the coast of China.

Diversity and distribution of three *Perkinsus* spp. haplotypes

A total of 43 haplotypes from four countries were detected in the *P. beihaiensis* global network (Figure 4). In this network, six shared haplotypes were found, including four haplotypes (H1, H2, H4, H6) shared by three countries and two haplotypes (H3, H5) shared by two countries (Figure 4). Five of six shared haplotypes were found in China. Eighty-six percent of haplotypes (37 of 43) was found in China, with 32 of the 37 haplotypes having been recovered only from China (Figure 4).

The haplotype networks of *P. olseni* showed that a total of 73 haplotypes were found from 11 countries (Figure 5). In this network, ten shared haplotypes were detected, including one haplotype (H10) shared by nine countries, two haplotypes (H3, H4) shared by eight countries, one haplotype (H9) shared by four countries, three haplotypes (H5, H6, H8) shared by three countries, and three haplotypes (H1, H2, H7) shared by two countries (Figure 5). Eight of the ten shared haplotypes were found in China. Sixty-seven percent of the haplotypes (49 of 73) was found in China, with 41 of the 49 haplotypes having been recovered only from China (Figure 5). All countries had at least one haplotype in common with at least one other country (Figure 5).

The *P. chesapeaki* global network analysis indicated that a total of 48 haplotypes were found from seven countries (Figure 6). In this network, three shared haplotypes were detected, including one haplotype (H1) shared by four countries (USA, Australia, France, China), one haplotype (H2) shared by three countries, and one haplotype (H3) shared by two countries (Figure 6). Two of three shared haplotypes were found in China. Fifteen percent of the haplotypes (7 of 48) was found in

TABLE 2 Chi-square test of PCR-prevalence of *Perkinsus* species among different regions.

X-squared	df	P.value	Species	Significance	Comparison subject
0.30	1	0.58	*Perkinus* spp		ECS VS SCS
3.83	1	0.05	*Perkinus* spp		ECS VS YBS
1.71	1	0.19	*Perkinus* spp		SCS VS YBS
16.08	1	0	*P. beihaiensis*	**	SCS VS ECS
55.85	1	0	*P. beihaiensis*	**	SCS VS YBS
14.01	1	0	*P. beihaiensis*	**	ECS VS YBS
0.15	1	0.70	*P. olseni*		ECS VS YBS
24.12	1	0	*P. olseni*	**	ECS VS SCS
20.88	1	0	*P. olseni*	**	YBS VS SCS
1.81	1	0.18	*P. chesapeaki*		SCS VS YBS
2.11	1	0.15	*P. chesapeaki*		SCS VS ECS
0.00	1	1.00	*P. chesapeaki*		YBS VS ECS

One asterisk corresponds to p-values between 0.01 and 0.05, and two asterisks correspond to p-values less than 0.01. ECS, East China Sea; SCS, South China Sea; YBS, Yellow and Bohai Sea.

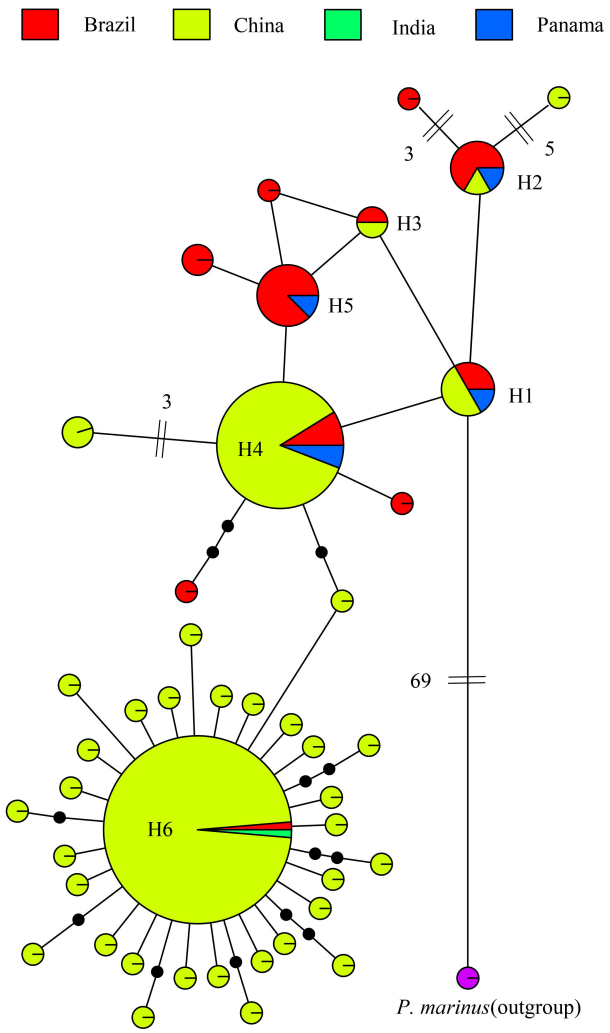

FIGURE 4
Haplotype networks of *Perkinsus beihaiensis* based on internal transcriber spacer sequence from China (this study) and additional sequences from GenBank that contained the sample location in their records. Colors indicate countries sampled. Neighboring haplotypes without double cross-bars indicate a single nucleotide mutation step or a gap. A nucleotide mutation step or gap of >1 between neighboring haplotypes is marked with double cross-bars, and the total numbers are indicated beside it. Black circles indicate inferred haplotypes not found in the sampled populations. Shared haplotypes are marked from H1 to H6 beside or inside the pie charts. The areas of the circles are proportional to the number of samples of each haplotype. *Perkinsus marinus* was used as the outgroup.

China, with five of the seven haplotypes having been recovered only from China (Figure 6).

Discussion

Previously, only two of seven known *Perkinsus* species (*P. olseni* and *P. beihaiensis*) had been reported in China, with *P. beihaiensis* as a new species first found in south China (Moss et al., 2008; Wu et al., 2011; Cui et al., 2018; Wu et al., 2019). *Perkinsus chesapeaki*, which was once a destructive pathogen causing decreased abundance and production of ecologically and economically important bivalve species in Chesapeake Bay, USA (McLaughlin et al., 2000), is first discovered carried by mollusks in coastal areas in China. Since its first report in North America, *P. chesapeaki* has been found invading Europe (Arzul et al., 2012), Australia (Dang et al., 2015), South America (Neto et al., 2016), and Asia (present study) in recent years. As commonly considered with other *Perkinsus* spp., this species was likely introduced into China through commercial transportation or introduction of their host molluscan species (Sheppard and Phillips, 2008; Pagenkopp Lohan et al., 2016). In China, three molluscan species—*S. subcrenata*, *M. lyrata*, and *S. constricta*—carried *P. chesapeaki*. *Perkinsus chesapeaki* was likely introduced

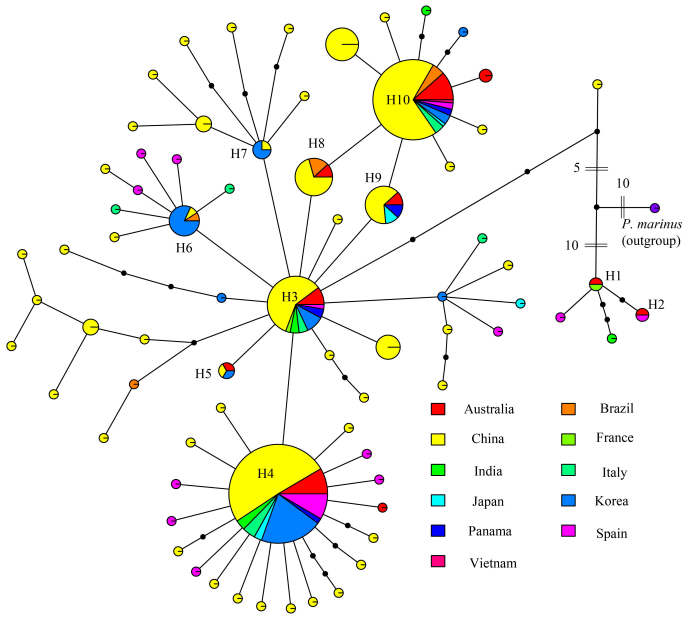

FIGURE 5
Haplotype networks of *Perkinsus olseni* based on internal transcriber spacer sequences. Colors indicate countries sampled. Neighboring haplotypes without double cross-bars indicate a single nucleotide mutation step or a gap. A nucleotide mutation step or gap of >1 between neighboring haplotypes is marked with double cross-bars, and the total numbers are indicated beside it. Black circles indicate inferred haplotypes not found in the sampled populations. Shared haplotypes are marked from H1 to H10 beside or inside the pie charts. The areas of the circles are proportional to the number of samples of each haplotype. *Perkinsus marinus* was used as the outgroup.

into China through the commercial transportation of these molluscan species or *Perkinsus*-susceptible ballast water from abroad.

The higher PCR-prevalences of *P. olseni* and *P. beihaiensis* suggest that these *Perkinsus* species have long been present in the coastal waters of China. Previous studies suggested that *P. olseni* carrying only occurred in the northern part of the China Sea, such as the YBS (Wu et al., 2011) or the neighboring sea in South Korea (Yang et al., 2012), while *P. beihaiensis* carrying only occurred in the southern part of the China Sea (Moss et al., 2008; Wu et al., 2018). In this study, however, *P. olseni* and *P. beihaiensis* carrying was found in three different coastal regions, indicating that these species have spread almost completely along the coastal waters of China, probably because they have sufficient reservoir hosts, and form a mature transmission route among different regions. The lower PCR-prevalence of *P. chesapeaki* at three different coastal regions suggests that this species may have been introduced to China more recently and lacks sufficient reservoir hosts.

Although *Perkinsus* spp. were previously regarded to have low host specificity (Pagenkopp Lohan et al., 2016), an increasing number of studies have shown that *Perkinsus* spp. have a tendency to infect the clam *R. philippinarum* and the oyster *C. hongkongensis*, with these two hosts exhibiting relatively higher *Perkinsus* species prevalence and abundance (Moss et al., 2008; Choi and Park, 2010; Yang et al., 2012; Wu et al., 2018; Yang et al., 2022). In fact, *Perkinsus* species may be less host specific, but the wide culture range, high culture density, and frequent commercial trade of these two hosts may be the main factors increasing the likelihood of *Perkinsus* spp. infection. For example, *R. philippinarum* was one of the most widely cultivated molluscan species on the mudflats along the coast of China, including the SCS, ECS, and YBS. A large proportion of fresh *R. philippinarum* cultivated in the SCS (i.e., Beihai, Qinzhou, Zhanjiang) was commercially transported to the city along the ECS (i.e., Shanghai, Xiamen) or YBS (i.e., Qingdao, Tianjin) for human consumption, which facilitated the transmission of *Perkinsus* spp. among different marine regions (Yang, 2016). *Perkinsus* spp. may be dispersed to other molluscan species mainly by co-existing with *Perkinsus*-infected *R. philippinarum* and *C. hongkongensis* in the same sea regions (Wu et al., 2018; Yang et al., 2022).

In this study, the widespread distribution of the three *Perkinsus* spp. along the coast of China suggests that the main factors influencing the biogeography of *Perkinsus* spp. is the culture mode and anthropogenic transportation of molluscan hosts, but not the host traits or specificity because *Perkinsus* spp. have low host specificity (Dungan and Reece, 2020). The movement of mollusks at different growth stages among different coastal waters is a routine operation in molluscan

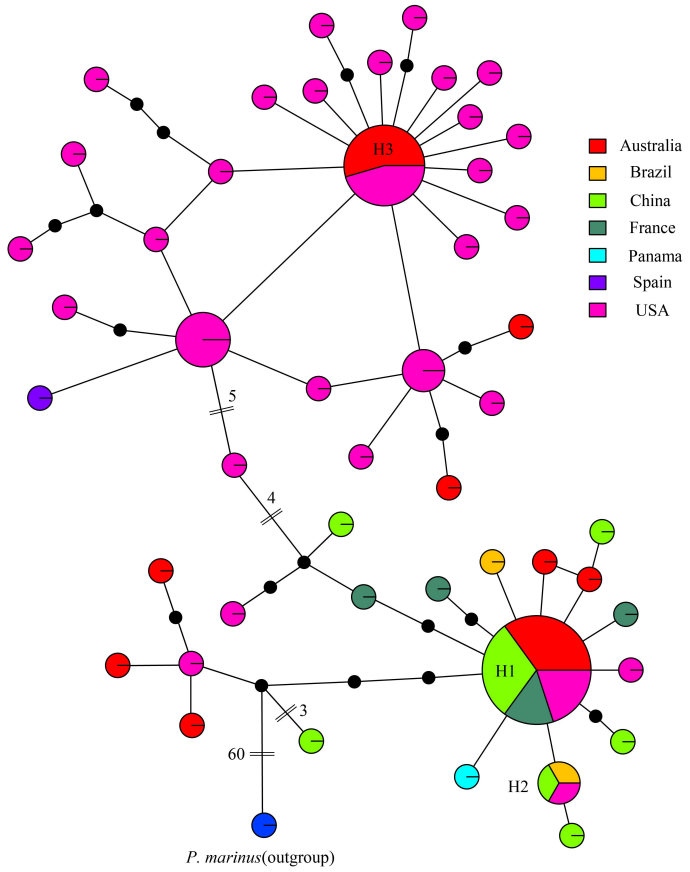

FIGURE 6
Haplotype networks of *Perkinsus chesapeaki* based on internal transcriber spacer sequence. Colors indicate countries sampled. Neighboring haplotypes without double cross-bars indicate a single nucleotide mutation step or a gap. A nucleotide mutation step or gap of >1 between neighboring haplotypes was marked with double cross-bars, and the total numbers are indicated beside it. Black circles indicate inferred haplotypes not found in the sampled populations. Shared haplotypes are marked from H1 to H3 beside or inside the pie charts. The areas of the circles are proportional to the number of samples of each haplotype. *Perkinsus marinus* was used as the outgroup.

culture in China. For example, most of the spats of the oyster *C. angulata* were hatched in Zhangzhou, Fujian Province (belonging to the ECS), but after approximately 2 months of culture, the spats were transported to coastal waters in Guangdong and Guangxi Province (belonging to the SCS) for further growth (Li et al., 2017). The long-distance transportation of commercial mollusks and broodstock also occurs regularly in China. The transportation of infected mollusks would thus contribute to the long-distance transmission of *Perkinsus* spp. in China. This movement may be the main transmission route for *Perkinsus* species across different coastal waters. Therefore, the detection and quarantine inspection of *Perkinsus* spp. during molluscan transportation are imperative for preventing the transmission and dispersal of *Perkinsus* spp. and containing outbreaks of perkinsosis in molluscan aquaculture (Cui et al., 2018).

Our results from the global haplotypes networks demonstrate that *P. olseni*, *P. beihaiensis*, and *P. chesapeaki* are three cosmopolitan parasite species, given that many shared haplotypes of these parasites were detected from different countries. We found that China had the greatest haplotype diversity of *P. beihaiensis* (86%) and *P. olseni* (67%) in the world, which suggests that these two species may originate from China, and have been widely transmitted and dispersed among molluscan species along the coast of China. Recent studies have demonstrated the higher tendency of *Perkinsus* infection with broader geographic range, lower prevalence, more diversified molluscan hosts, and more diversified *Perkinsus* haplotypes (Pagenkopp Lohan et al., 2016; Pagenkopp Lohan et al., 2018; Luz et al., 2018; Wu et al., 2018; Itoh et al., 2019; Luz Cunha et al., 2019). In the 21st century, molluscan mass mortality events caused by *Perkinsus* infection seldom occurred anywhere in the world. In the coastal waters of China, our team has been consistently monitoring *Perkinsus* infection variation in mollusks over the past decade (Cui et al., 2018; Wu et al., 2018; Wu et al., 2019; Yang et al., 2022), but we have not

found any mass mortality of mollusks related to *Perkinsus*. Similar to our previous study, we found that *Perkinsus* infection in mollusks was characterized by no obvious gross signs, medium or low prevalence, low host specificity (Wu et al., 2018; Yang et al., 2022), and low abundance (Cui et al., 2018). We hypothesize that susceptible molluscan hosts (i.e., *R. philippinarum*) have improved the immunity and tolerance to *Perkinsus* infection after an extended period of competition and adaptation, thus lowering the prevalence or abundance of *Perkinsus* spp. and decreasing the chance of mass mortality. In response, *Perkinsus* spp. would target some other new molluscan species to maintain its population through continued survival and reproduction. The successful colonization of new mollusks not only contributes to the transmission and dispersion of *Perkinsus* spp., but also promotes the evolution of *Perkinsus* spp., thus increasing their genetic diversity. New molluscan hosts may also burden a large proportion of *Perkinsus* spp. that infected susceptible molluscan hosts, thus diluting the abundance of *Perkinsus* spp. in susceptible molluscan hosts and lowering the incidence of perkinsosis.

Conclusion

Our study reports, for the first time, the presence of *P. chesapeaki* along the coastal waters of China. Three cosmopolitan parasite species—*P. olseni*, *P. beihaiensis*, and *P. chesapeaki*—can be detected in most molluscan species, suggesting that they are widespread along the coastal waters of China. *Perkinsis olseni* is prominent in the YBS and ECS, while *P. beihaiensis* is prominent in the SCS. Most molluscan species carried *Perkinsus* spp. with medium or low levels of PCR-prevalence (<30%). Three *Perkinsus* spp. possess high levels of ITS haplotypes, some of which are shared among many countries. The main factors shaping *Perkinsus* species biogeography are culture mode and anthropogenic transportation of molluscan species, but not the host traits or specificity. The far higher PCR-prevalence of *Perkinsus* spp. in *R. philippinarum* and *C. hongkongensis* suggests that *Perkinsus* spp. may be transmitted and dispersed to other molluscan species through the transportation of *Perkinsus*-carried *R. philippinarum* and *C. hongkongensis* species. *Perkinsus* carrying tended to be generally linked with a broader geographic range, lower prevalence, more diversified molluscan hosts, and more diversified *Perkinsus* haplotypes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/- accession numbers MT908881 – MT908909.

Ethics statement

The animal study was reviewed and approved by South China Sea Fisheries Research Institute Academic Committee.

Author contributions

LY: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review and editing, Funding acquisition, Project administration. LW: Investigation, Writing - original draft, Writing - review and editing. JL: Resources, Software, Validation. TY: Investigation, Validation, Visualization. JW: Investigation, Validation, Writing - original draft. SS: Investigation, Writing - original draft. GY: Writing - review and editing. WZ: Investigation, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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