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Withering syndrome in the abalone *Haliotis diversicolor supertexta*

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ABSTRACT: Abalone aquaculture is a small but growing industry in Thailand and is based on both the exotic *Haliotis diversicolor supertexta* and the native *H. asinina*. Withering syndrome (WS) in abalone is caused by an infection with the Rickettsia-like organism (RLO) '*Candidatus* Xenohaliotis californiensis' and has been spread to many countries globally. The present study reports the first observation of the WS-RLO agent in the small abalone, *H. diversicolor supertexta* in Thailand, Taiwan (ROC) and the People's Republic of China (PRC). Under light microscopy, the RLO was observed as intracytoplasmic inclusions within epithelial cells lining the post-esophagus and, to a minor extent, the intestine of *H. diversicolor*. Under transmission electron microscopy, inclusions were characterized as colonies of rod-shaped bacteria, 200 × 1800 nm in size, within a vesicle in the cytoplasm of the infected cell. The RLO from the small abalone bound with WS-RLO-specific *in situ* hybridization probes and was amplified by polymerase chain reaction (PCR), using primers designed from the 16S rDNA sequence of the original WS-RLO from California, USA. The PCR product of RLO samples from both the PRC and Thailand showed extremely high identity with the California WS-RLO (100 and 99%, respectively). These data combined with the history of abalone movements for aquaculture purposes indicate that RLOs observed in Thailand, Taiwan and the PRC are the WS-RLO that originated from California.

KEY WORDS: *Haliotis diversicolor* · '*Candidatus* Xenohaliotis californiensis' · 16S rDNA · Abalone · Withering syndrome · Thailand · Taiwan · People's Republic of China · California

INTRODUCTION

Culture of the small tropical abalone *Haliotis diversicolor supertexta* is an emerging commercial industry in several Asian countries such as in Taiwan, Thailand and southern China because of its high market potential. The first commercial abalone farm was started in 1996 in southern Thailand on Phuket Island. Currently, most of the abalone farms (n = 5 to 6) in Thailand culture the native species *H. asinina*, as it has the highest meat yield of the 3 native Thailand haloitids (Klinbunga et al. 2004); another 2 or 3 farms culture the imported species *H. diversicolor supertexta* (Jarayabhand et al. 2009) due to its high market demand (P. Jarayabhand, Chulalongkorn University, Thailand, pers. comm.). Thailand abalone production is currently low, with an estimated annual yield of from 60 to 100 metric tonnes (P. Jarayabhand pers. comm.). As with many developing aquaculture sectors, growth of the industry is accompanied by the movement of animals among locations, which can result in the transfer of pathogens along with the target host species. It is important to know if potential pathogens, such as the

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rickettsial agent of withering syndrome (WS) are present in abalone under culture. Prior to the present study, the infection of Asian abalone species by Rickettsia-like organisms (RLOs) had not been observed or described; however, such knowledge is needed for the successful development and expansion of abalone aquaculture in Asia.

WS is a catastrophic disease, caused by the intracellular RLO '*Candidatus* Xenohaliotis californiensis' (Friedman et al. 2000). WS affects a variety of abalone species and was first observed in populations of wild black abalone *Haliotis cracherodii* in the mid-1980s (Haaker et al. 1992). Affected black abalone had an atrophied foot muscle and high mortality rates, especially in populations off southern California, USA (Haaker et al. 1992). The disease progressively spread throughout southern and central California (Haaker et al. 1992, Altstatt et al. 1996, Moore et al. 2000, Friedman & Finley 2003, Miner et al. 2006). By 1992, WS was associated with population declines on 6 of the 8 Channel Islands, with losses of up to 99% of *H. cracherodii* (VanBlaricom et al. 1993, Tissot 1995). These losses led, in part, to an initial closure of the California black abalone fishery in 1993, followed by total closure in 1997 (Haaker et al. 2001). Losses due to WS vary among host species from extremely high losses in white abalone (Friedman et al. 2007) to moderate losses in red abalone (Moore et al. 2000) and low to moderate losses in green and pink abalones (Tinajero et al. 2002, Vilchis et al. 2005, Moore et al. 2009). In Baja California, Mexico, 32.5% of pink and 27% of green abalones examined had clinical signs of WS (Tinajero et al. 2002). However, green abalone only showed signs of WS when held at elevated temperatures, e.g. 25°C (Garcia-Esquivel et al. 2007), but not when held at 18°C, a temperature at which black and red abalones typically succumb to WS (Friedman et al. 2002, Moore et al. 2009).

We conducted a pilot survey of the small abalone from several Asian countries in an attempt to discern if the WS-RLO was present in the sampled populations. The present study reports on the first case of observed WS-RLO infection in *Haliotis diversicolor supertexta*.

MATERIALS AND METHODS

Specimens. Thirty-six small abalone *Haliotis diversicolor supertexta* measuring 11.09 ± 4.4 g in body weight (BW) and 39.4 ± 5.8 mm in maximum shell length were collected in March and April 2006 from commercial farms in southern Thailand, in HuiZhou City, GuangDong Province, People's Republic of China (PRC), and in TouCheng Township, YiLiang County, Taiwan, as outlined in Table 1.

Table 1. *Haliotis diversicolor.* Diagnostic testing results for withering syndrome-Rickettsia-like organism (WS-RLO) detection in abalone, using 4 detection methods

Method	Origin	No. tested	No. positive
Light microscopy	Thailand	22	12
	PR China	6	3
	Taiwan	8	4
In situ hybridization	Thailand	7	7
	PR China	3	3
	Taiwan	4	4
Polymerase chain reaction	Thailand	22	12
	PR China	6	4
	Taiwan	8	6
Transmission electron microscopy	Thailand	5	5

The abalone had been reared in ambient seawater ranging from 27 to 29°C. All individuals, which appeared normal by gross examination, were sacrificed by severing of the cerebral and pedal ganglia with a scalpel blade prior to excision of post-esophagus, digestive gland, intestine and foot muscle. The tissues were preserved and processed for light microscopy (LM), *in situ* hybridization (ISH), transmission electron microscopy (TEM) and for polymerase chain reaction (PCR) as described below.

Histology and ISH. The number of samples processed for each method is shown in Table 1. For LM and ISH, the tissues were processed according to Friedman et al. (1997, 2002) and Antonio et al. (2000), respectively. Briefly, tissues were fixed in invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h, transferred to 70% ethanol, and processed for routine paraffin histology. For LM, deparaffinized tissue sections (5 µm) were stained with hematoxylin & eosin (H&E) and viewed by light microscopy. RLO infections were rated at 200× magnification according to the logarithmic scale of Friedman et al. (1997), in which no visible RLO is rated as 0, 1 to 10 RLO inclusions per field of view is rated as 1, 11 to 100 inclusions per field is rated as 2 and >100 inclusions is rated as 3. For ISH, the sections were placed on positively charged microscopic slides (Fisher Scientific) and processed according to Antonio et al. (2000), with the exception that we employed a probe labeled with digoxigenin (DIG) via PCR according to the manufacturer's instructions (PCR DIG probe Synthesis Kit, Roche Applied Sciences). Tissue sections from known WS-RLO-infected red abalone *Haliotis rufescens* served as positive controls. Tissue sections identically treated, but lacking the DIG-labeled probe, served as negative controls. The

sections were examined under LM; cells showing a hybridized RLO-DNA probe displayed dark-blue to dark-purple precipitates.

TEM. Tissues were preserved in 4% glutaraldehyde in 0.15 M Millonig's phosphate buffer (pH 7.4) supplemented with 1% NaCl and 0.5% sucrose overnight at 4°C, rinsed twice with Millonig's buffer and post-fixed in 1% OsO_4 in the same buffer at 4°C for 1 h. Samples were then washed in Millonig's buffer and dehydrated at room temperature through a graded series of ethanol. Dehydrated tissues were incubated in propylene oxide and embedded in Epon (EMS). Semithin sections were stained with 1% toluidine blue (in 1% aqueous sodium borate) and observed under LM for the presence of potential lesions. Subsequently, ultrathin sections were stained with uranyl acetate and lead citrate and observed by TEM (Hitachi H7500).

PCR and sequence analysis. Amplification of RLO DNA was used as a proxy for RLO infection. A 160 bp fragment of the 16S rDNA of the RLO was amplified in 20 µl reactions containing 2 µl template, 1.6 U of *Taq* DNA polymerase in 1× Storage Buffer A (Promega), 1.5 mM $MgCl_2$, 200 µM dNTPs, 400 ng ml^{-1} BSA, and 0.5 µM of primers RA 3-6 and RA 5-1 (Andree et al. 2000). Cycle conditions included an initial denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 62°C for 30 s and 72°C for 30 s and a final elongation step of at 72°C for 10 min. Amplified fragments were separated on a 1% agarose gel that contained ethidium bromide and visualized on an ultraviolet transilluminator. Amplified PCR fragments from 8 Thailand abalone and 4 PRC abalone were directly sequenced at the University of Washington's Department of Biochemistry DNA Sequencing Facility (Seattle, Washington) using a 3730XL DNA analyzer (Applied Biosystems). Consensus sequences for Thailand samples and PRC samples were obtained using a Sequencher software program (Genecodes). Consensus sequences were aligned with the USA sequence available from GenBank (Accession No. AF133090) via multiple sequence alignments using ClustalW (Thompson et al. 1994).

RESULTS

Histology

Basophilic (violet to purple) RLO inclusions were observed in 19 of 36 abalone *Haliotis diversicolor* examined by histology; approximately 50% of the abalone examined from each country was infected (Table 1). RLO infection intensities in the post-esophagus and intestine were rated as Grade 2 (19.9 ± 6.3 inclusions per 20× field of view), which were considered light to moderate infections (Table 1, Fig. 1a, b; Friedman et al. 1997). Only one abalone had RLO inclusions in the intestine (Fig. 1c). Inclusions were situated apical to the nucleus of the host cells, were mostly spherical or oval in shape, and measured from 5 to 30 µm in size. Presence of inclusions caused an expansion of the infected epithelial cell, the microvilli of which retained normal features (Fig. 1b). The intestine of the RLO-infected *H. diversicolor supertexta* revealed an intact epithelial cell structure containing RLOs in the cytoplasm. The myofibers of all foot muscles examined were abundant and tightly packed, with little or no intervening space or signs of atrophy (Fig. 1d).

ISH

The ISH revealed positive hybridization signals in the epithelial cells of the post-esophagus in all 14 abalone tested that also had histological signs of WS-RLO infection (Table 1, Fig. 2a). One abalone from Thailand also showed ISH evidence of infection in the intestine (Fig. 2b). ISH-labeled inclusions ranged from 1 to 2 µm to >15 µm in size and were clearly distinguished from the light background. In most infected cells, only 1 colony was observed; however, 2 to 3 colonies were occasionally observed within a single host cell. The positive control, tissue sections from a known RLO-infected red abalone *Haliotis rufescens*, clearly demonstrated the presence of a positive reaction in the cytoplasm of the epithelium (Fig. 2c). Tissue sections from RLO-infected *H. diversicolor supertexta* fully processed through ISH, but lacking the 16S rRNA probe, did not produce the characteristic DIG-brown precipitate and were considered negative (Fig. 2d).

TEM

Examination of thin sections of post-esophagus tissues from 5 infected *Haliotis diversicolor supertexta* that contained intra-cytoplasmic inclusions (Table 1) revealed the presence of abundant rod-shaped microorganisms with mean (±SD) lengths of 0.4 ± 0.14 to 1.7 ± 0.32 µm (Fig. 3). The bacteria were localized within membrane-bound cytoplasmic vacuoles of the host cell (Fig. 3b) and exhibited ultrastructural characteristics of prokaryotic cells, including a trilaminar cell wall, an electron-dense periplasmic ribosome zone and a nucleoid (Fig. 3c). The cell wall (~50 nm in width) was composed of the outer membrane surface, the middle electron-lucent layer and the inner membranous layer, which was thicker than the outer layer; a peptidoglycan layer was not visible.

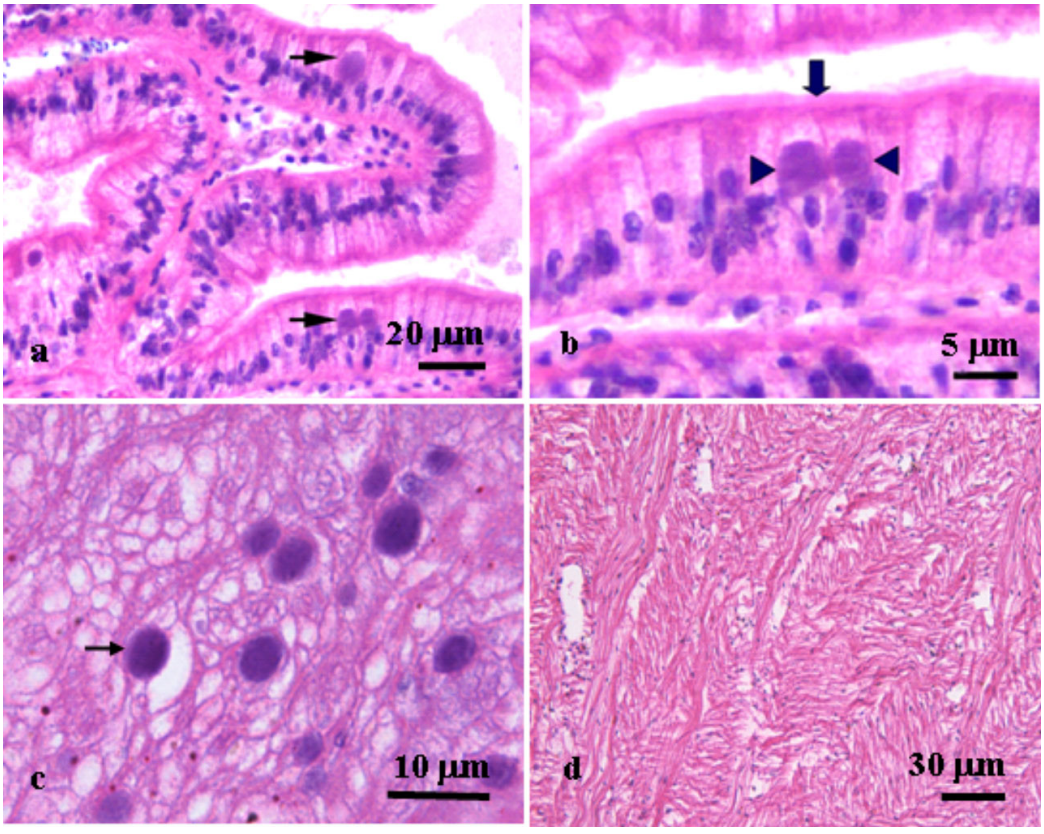


Fig. 1. *Haliotis diversicolor supertexta*. Photomicrographs of the post-esophagus of abalone from Thailand showing (a) basophilic intra-cytoplasmic inclusions (arrows), which (b) are magnified (arrowheads) and show the intact microvilli of the epithelial cells (arrow). (c) Inclusions were also observed in an intestine of 1 out of 19 individuals (arrow). (d) Foot muscle shows normal muscle fibers

PCR and sequence analysis

PCR and sequence analyses in the abalone provided further confirmation of the presence of the WS-RLO in 22 out of 36 abalone tested (Table 1, Fig. 4a). DNA from all the individuals that contained morphological evidence of infection (by LM, TEM and ISH) and an additional 3 samples lacking visible RLO inclusions (22 abalone total) amplified the expected 160 bp product. The RLOs from China and Thailand abalones showed 100 and 99% homology, respectively, with the California WS-RLO (Fig. 4b).

DISCUSSION

Using methods outlined by the World Animal Health Organization (OIE 2006), we have reported the first observation of the WS-RLO in asymptomatic *Haliotis diversicolor* in Thailand, Taiwan and the PRC. The morphological and genetic traits of the RLOs from these countries are indistinguishable from the WS-RLO described from California. The Asian and California RLOs are rod-shaped, measure approximately 300 to 400 nm by 1500 to 1700 nm, contain ribosomes adjacent to the cell perimeter, and are delimited by a trilaminar cell wall that lacks an apparent peptidoglycan layer (Friedman et al. 2000). Using LM, the RLO inclusions stained a violet to purple color and were situated apical to the nucleus (Friedman et al. 2000). Genetic similarity was high, as evidenced by binding of the WS-RLO ISH probe (Antonio et al. 2000) with the Asian RLO combined with from 99 to 100% similarity of the amplified 160 bp fragment using published primers specific to WS-RLO (Andree et al. 2000). The 1% difference (1 of 99 bp excluding the primers) in the sequence that was observed between the RLO DNA from Thailand and the RLO from California may be due to polymorphisms in this gene or mutations in the RLO from Thailand compared to the original strain.

We provide evidence that the origin of the RLOs found in our Asian abalone is likely California, USA; the transfer occurred putatively when red abalone seed from a RLO-infected California hatchery was sold to China (R. Gordon, Fish Tech, pers. comm.) in the mid-1990s, prior to the development of molecular diagnostic

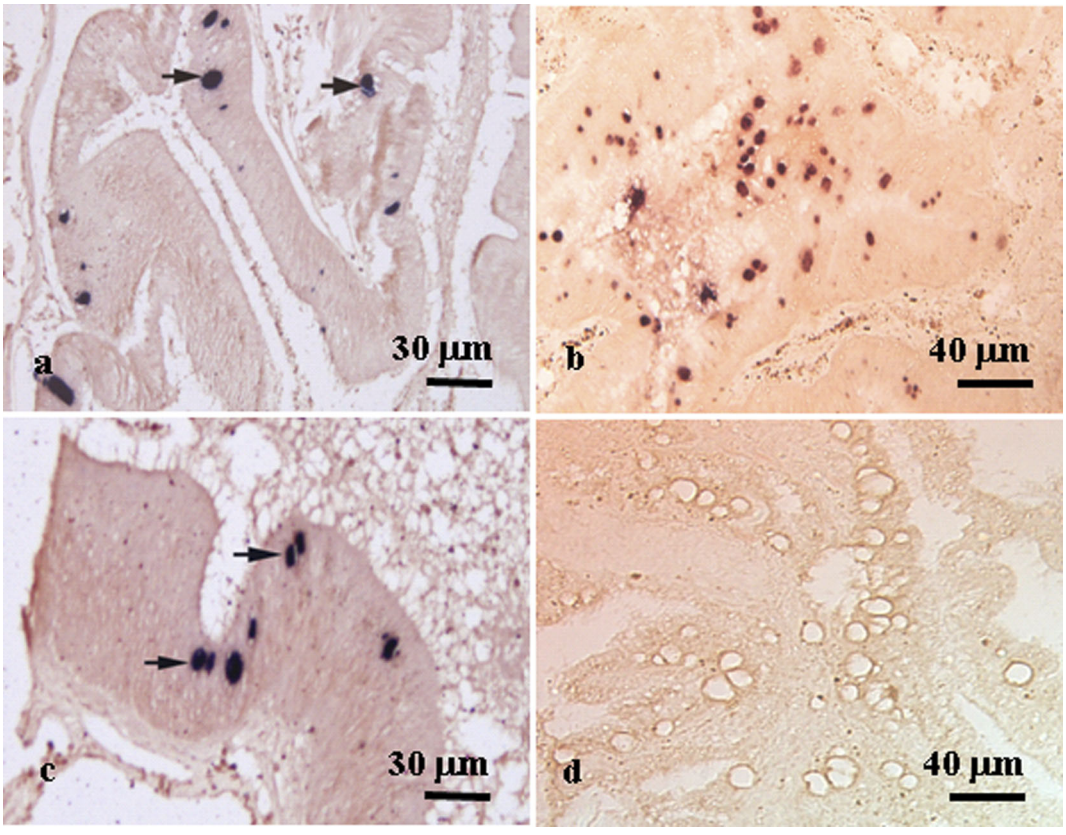


Fig. 2. *Haliotis diversicolor supertexta*. Photomicrographs of the (a) post-esophagus and (b) intestine of abalone from Thailand processed through *in situ* hybridization showing positive Rickettsia-like organism (RLO)-primer hybridization as dark brown inclusions. (c) Post-esophagus tissue of RLO-infected *H. rufescens* shows a positive reaction (arrows). (d) Post-esophagus section from RLO-infected *H. diversicolor supertexta* fully processed through *in situ* hybridization, except that the 16S rRNA probe was omitted, illustrates a negative reaction

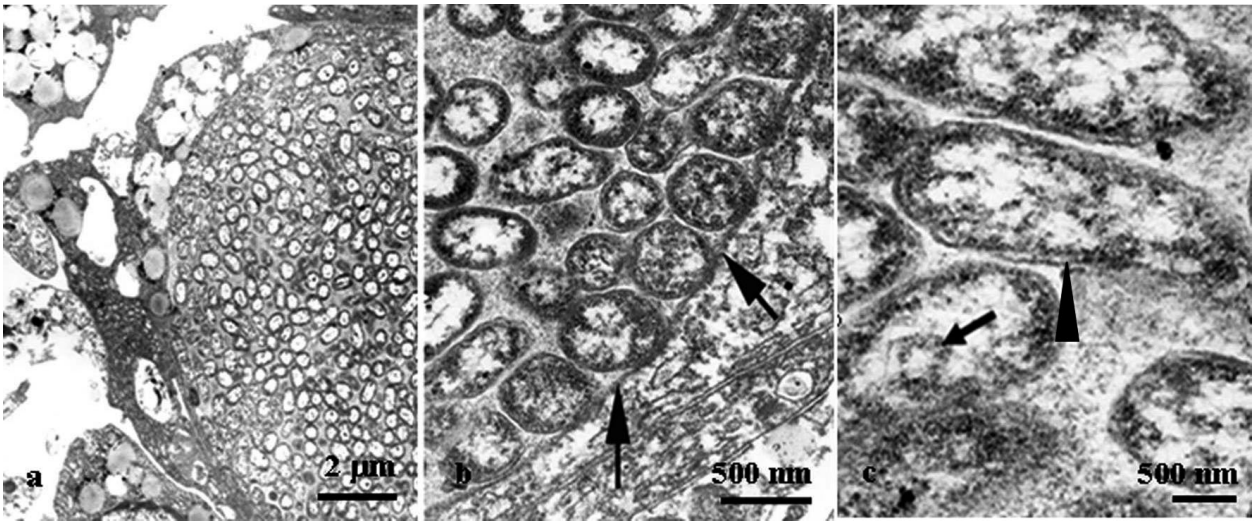
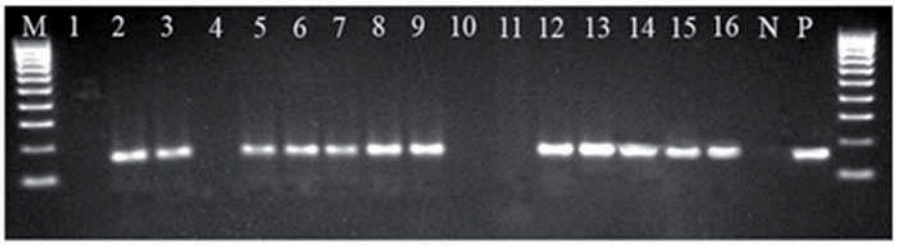


Fig. 3. *Haliotis diversicolor supertexta.* Transmission electron micrograph of the post-esophagus of abalone from Thailand showing (a) numerous rod-shaped bacteria in cross section, which (b) were localized within membrane-bound vacuoles (arrows). (c) Cell walls of the Rickettsia-like organisms (RLOs) are composed of 3 layers: outer and inner electron-dense layers separated by an electron-lucent layer (arrowhead). A nucleoid structure was observed in some of the bacteria (arrow)

a



b

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PR China    CGGGTGAGTAATGCTTGGAAATCTACTCAGAAGACATGAATAACTATCAGAAATGGTAGC 60
GenBank     CGGGTGAGTAATGCTTGGAAATCTACTCAGAAGACATGAATAACTATCAGAAATGGTAGC 60
Thailand    CGGGTGAGTAATGCTTGGAAATCTACTCAGAAGACATGAATAACTATCAGAAATGATAGC 60
            ************************************************************ ****

PR China    TAATACAAGATAATACCCTGAGGGGGAAAGGTTTATTCC 99
GenBank     TAATACAAGATAATACCCTGAGGGGGAAAGGTTTATTCC 99
Thailand    TAATACAAGATAATACCCTGAGGGGGAAAGGTTTATTCC 99
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Fig. 4. *Haliotis diversicolor supertexta.* (a) Agarose gel electrophoresis of the amplified products from the post-esophagus of abalone, using primers specific for the 16s rRNA of the withering syndrome-Rickettsia-like organism (WO-RLO). Lane M: 1000 bp DNA molecular marker; Lanes 1 to 16: samples; Lane N: negative control; Lane P: RLO-infected *H. rufescens*. (b) Sequences of specimens from PR China and Thailand were aligned with those from the USA (available from GenBank; AF133090) via multiple sequence alignments using ClustalW. Asterisks illustrate consensus among sequences

tools. Abalone were, in turn, moved from China to Taiwan in 2000 (B. Withyachumnarnkul pers. comm.). Seed from this same California hatchery has previously been identified as the inadvertent vector for introducing WS-RLO into a natural population in northern California during a restoration study (Rogers-Bennett & Pearse 1998, Friedman & Finley 2003). Unlike the infections in many abalone species reported to date, the WS-RLOs in our Asian abalone samples were mainly detected in low to moderate numbers within post-esophageal epithelia and, in one case, in the intestine, but not in the epithelium of the digestive gland (Gardner et al. 1995, Antonio et al. 2000, Friedman et al. 2000). Low-level infections, particularly in seed abalone, may be difficult to diagnose (Friedman et al. 2007) and may account for the introduction of WS-RLOs via seed abalone from California, despite annual histological examinations of 60 seed (designed to detect parasites at 5% or greater prevalence; OIE 2006). Our data highlight the imperative for health examinations prior to movement of animals among locations, particularly to distant locations; examinations should be conducted at the 2% prevalence detection level (n = 150 individuals) and should include both microscopic (histology) and molecular tests (e.g. PCR) when available. Whether or not health examinations were conducted prior to movement of abalone within Asia is not known.

Although WS-RLO-infected *Haliotis diversicolor supertexta* did not show gross signs of clinical disease in this pilot study and none of the farms contacted reported unusual losses, health examinations prior to animal movements are still needed for successful expansion of aquaculture. Whether a lack of digestive gland metaplasia is a sign of recent infection, host resistance, or due to physiological limitations (e.g. temperature limited) of the RLO is not known at this time. When the *H. diversicolor supertexta* examined in this pilot survey became infected is not known, but infection may have occurred just prior to the examination; however, PCR analysis of this same population of abalone sampled at an earlier life stage (0.35 g BW) also revealed the presence of the infection (T. Wetchateng et al. unpubl. data). Therefore, it is possible that the abalone had been infected by RLOs for at least 9 mo, but were still asymptomatic. The WS-RLO is known to infect juvenile to adult abalones (Haaker et al. 1992, Friedman et al. 1997, 2000, 2007), and all post-larval life stages appear susceptible to the infection (J. D. Moore & C. S. Friedman unpubl. obs.). The incubation period of WS varies with temperature, but typically ranges from 3 to 7 mo, and mortality typically occurs from 1 to 2.5 mo after the onset of visible clinical signs (Friedman et al. 1997). In addition, RLO replication and the associated disease development are enhanced at temperatures >17°C (18 to 25°C), suggesting a thermal induction of WS and its RLO (Moore et al. 2000, Braid et al. 2005, Garcia-Esquivel et al. 2007). Metaplasia is commonly observed in WS-RLO-infected abalones held at water temperatures >18°C (Friedman et al. 1997, 2000, 2007). Natural resistance of *H. diver-*

sicolor supertexta to RLO infection, as compared to most of the highly susceptible cold-water species reported from California, USA (Friedman et al. 2000, 2002, 2007, Moore et al. 2001, 2002), may be another explanation for this observation. Finally, temperatures used to culture *H. diversicolor supertexta* (27 to 29°C) may be near the upper limit of the WS-RLO, thereby limiting its ability to cause host metaplasia, advanced infections and clinical disease. Characterization of the thermal tolerance range of the WS-RLO, an obligate intracellular parasite, has been limited by the thermal range of the host abalone species. Infections in *H. diversicolor supertexta* provide an opportunity to further explore the thermal range of the WS-RLO. Further studies on the WS-RLO and *H. diversicolor supertexta* are needed to elucidate the host–parasite relationship.

In conclusion, although the original source of the WS-RLO in *Haliotis diversicolor supertexta* is not known, we suspect, based on sequence homology and transport history, that it originated from California. Although California abalone were given annual histological health examinations, histology has been shown to be less sensitive than molecular tools in identifying individuals potentially infected with the WS-RLO (Friedman et al. 2007); this may be especially true in small seed abalone. In addition, the abalone reared in Thailand were purchased from Taiwan (B. Withyachumnarnkul pers. comm.), where the cultivation of this species has been practiced for many years, further illustrating the potential anthropogenic movement of this pathogen. Thus, extensive, high-precision health examinations of *H. diversicolor supertexta* should be legally required prior to movement between sites within and outside Thailand, to prevent the spread of this disease as suggested by the OIE (2006) to control aquatic animal diseases (OIE 2006).

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