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Research Article

Invasive Apple Snails in Wetlands of Selangor, Malaysia: Species, Distribution, and Ecological Associations

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Abstract
Apple snails in the genus *Pomacea* are among the worst invasive species in Southeast Asia. Our objectives were to survey a selection of different wetlands in Selangor for *Pomacea,* verify which species of *Pomacea* occurred in that location, and assess basic environmental parameters associated with their presence and relative abundance. Aquatic parameters including pH and concentrations of selected electrolytes were measured at 25 wetland sites distributed among eight localities in Selangor. DNA from snails collected at each locality was extracted and the mitochondrial cytochrome c oxidase subunit I (COI) was sequenced. We detected two of the most successful invaders of this genus: *P. canaliculata* was found in five localities and *P. maculata* in two. Both pH and calcium ion concentrations were negatively associated with *Pomacea* presence. *Pomacea* were absent in brackish wetlands with high pH and calcium concentrations reflecting possible physiological intolerance or that dispersal into these habitats has yet to occur. *P. maculata* is reported to tolerate pH as low as 4.5-6; thus most freshwater wetlands in Selangor and most of Malaysia can potentially be invaded. *Pomacea canaliculata* and *P. maculata* have demonstrated remarkable capacity for depleting aquatic macrophytes and may cause rapid changes in aquatic plant communities with potential impacts to wetland state and function. Public awareness and environmentally safe recommendations to mitigate the reproduction and spread of this invasive snail is needed for protecting the biodiversity and health of natural wetlands.

Keywords: *Pomacea canaliculata*; *Pomacea maculata*; environmental parameters; *cox*1 gene; invasive species; wetlands; Selangor

Introduction
Some of the most aggressive invaders in freshwater systems are ampullariids that are indigenous to humid tropical regions of South and Central America (Qiu & Kwong, 2009). The most species-rich genus in the Ampullariidae is

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Pomacea with 96 nominal species, although the actual number of species is estimated at 50 (Hayes et al., 2015). Certain species of *Pomacea* are notorious for their voracious appetite for macrophytes, causing significant damage to rice fields and other wetland agriculture (Horgan et al., 2014). *Pomacea canaliculata* is listed among the world's 100 worst invasive species (Lowe et al., 2000), but reports of this species have been frequently confounded with its morphologically similar congener, *P. maculata* (Rawlings et al., 2007; Matsukara et al., 2008; Hayes et al., 2012). Thus, reports of damage by "golden apple snails", which were previously thought to be one species (Hayes et al., 2008; Hayes et al., 2012) may have alluded to either *P. canaliculata* or *P. maculata*. Unless specified, use of the name *Pomacea* in the remainder of this manuscript will refer to these two invasive species.

Pomacea was introduced to Malaysia in the late 1980's (Cowie, 2002), and was later identified as *P. canaliculata* using molecular diagnosis (Hayes et al., 2008). The snails may have been introduced to Southeast Asia with the purpose of becoming a local food and export item. Eventually escaping into agricultural wetlands, the snails quickly spread through the Asian rice irrigation system into natural wetlands, and thrived in these new habitats (Naylor, 1996). Agricultural impacts by invasive species of *Pomacea* have received far more attention to date than its spread and impacts on wetland ecosystems. However, a few studies indicate dramatic losses to wetland macrophytes (Carlsson et al., 2004), shifts in the state and function of natural wetlands (Horgan et al., 2014), and possible cascading effects including the decline and extirpation of native ampullariids (Cowie, 2000; Horgan et al., 2014). In Malaysia, two species of *Pomacea* have been reported: *P. canaliculata* (Yahaya et al., 2006; Salleh et al., 2012) and *P. maculata* (Arfan et al., 2014), but to our knowledge, there was no molecular confirmation of the species.

Wetlands provide ecosystem services, such as water purification, nutrient cycling, and flood control, of tremendous ecological and economic significance (Sather & Smith, 1984). Costanza et al. (1997) estimated the per hectare economic value of wetlands to be twice that of lakes and rivers, four times that of coastal ecosystems, and seven times that of tropical forests. Wetland macrophytes play an important role in water purification through erosion control and pollutant retention. A diversity of flora and fauna rely on wetland habitats for survival. Because apple snails feed predominantly on fresh macrophytes, have high growth rates, large body masses and high reproductive output, they can cause rapid changes to the macrophyte community structure, including increased water turbidity and shifts in wetland ecosystem function (Sheldon et al., 2003; Horgan et al., 2014). In Southeast Asia, wetlands invaded by *P. canaliculata* shifted from macrophyte dominance to phytoplankton-dominant, which resulted in higher levels of aquatic nitrogen and phosphorus (Carlsson et al., 2004).

Knowledge of the distribution and species of *Pomacea*, including associated environmental factors is needed to predict the types of habitats in Peninsular Malaysia that are vulnerable to invasion, and for developing effective management strategies (Rawlings et al., 2007; Hayes et al., 2012; Horgan et al., 2014). Morphologically, some species of *Pomacea* demonstrate high intraspecific variability and interspecific overlap, making identification to species difficult (Hayes et al., 2012). Using a single-locus genetic approach to confirm species identity, we provide the first information on the species of *Pomacea*, their distribution, and associated environmental parameters in natural and agricultural wetlands in Selangor, Malaysia. Specifically we asked the following questions: 1) Which species of *Pomacea* occur in Selangor; and 2) Which aquatic parameters are associated with the presence and abundance of *Pomacea spp.* in Selangor wetlands.

Materials and Methods

Study Area

Selangor is the most populous state in Malaysia with approximately 800,000 ha of land and 5.8 million people (Department of Statistics Malaysia, 2008-2015). Monthly mean temperature and rainfall ranges from 23 °C to 33 °C and 90 mm to 300 mm. Northeast and eastern Selangor is covered with undulating hills, whereas the central and coastal regions are relatively flat (Abdullah & Nakagoshi, 2008). These relatively flat regions support abundant wetland habitats and abandoned tin mine lakes, including the northern coastal region in Sekinchan, which supports intensive rice agriculture. Based on shell morphology, Arfan et al. (2014) reported the presence of *P. canaliculata* and *P. maculata* in rice fields in Peninsular Malaysia (Arfan et al., 2014); thus these, including other species of *Pomacea*, could potentially occur in other types of wetland habitats.

Fieldwork

Twenty-five sampling sites distributed among eight localities (Figure 1) were surveyed for *Pomacea* presence/absence and measured for a set of aquatic

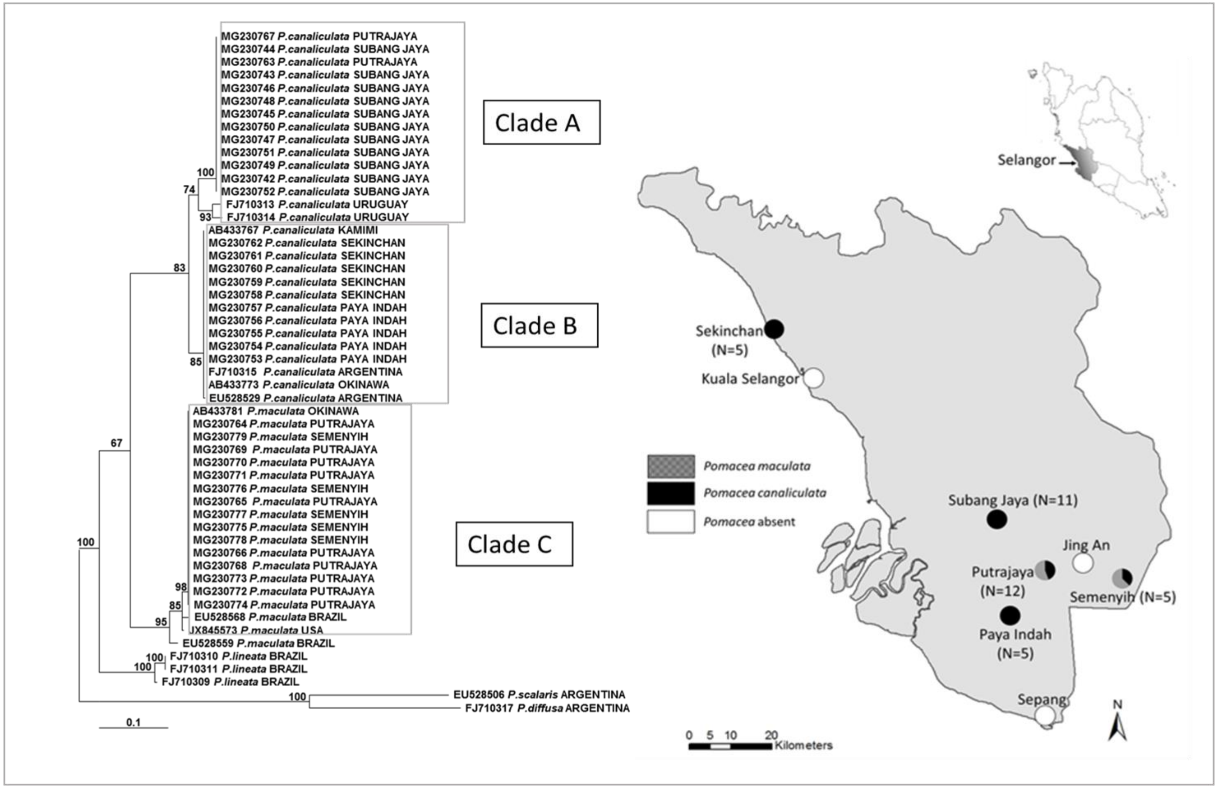

Figure 1. Sampling localities for *P. canaliculata* and *P. maculata* in Selangor, Peninsular Malaysia, September – November 2016. Phylogenetic tree was based on the mitochondrial *cytochrome c* oxidase subunit I (COI) gene. Node values represent 1000 bootstrap replicates (> 50%) under maximum likelihood. *Pomacea scalaris* and *P. diffusa* were selected as outgroup taxa. GenBank accession numbers for all individuals sequenced in this study are included.

variables, namely pH, salinity, conductivity, nitrate, calcium and potassium, that we considered important for aquatic snails. Population density of aquatic organisms tends to decrease as pH becomes more acidic, suggesting low pH levels affect biodiversity and productivity (Bemvenuti et al., 2003). Aquatic pH is one of the principle variables affecting distribution and survivorship of *Pomacea* (Ito 2002, 2003; Byers et al., 2013; Pierre, 2015). *Pomacea* distribution and physiological functions are limited by high salinity (Costil et al., 2001). Conductivity provides an insight to the total amount of ions in water that allows electrical flow (Horiba Scientific, 2016). High nitrate, phosphate and potassium levels indicate potential eutrophication that leads to algal proliferation and eventually low oxygen levels in water bodies, which may not be suitable environments for oxygen-dependent organisms, and could possibly limit survival (Smith et al., 1999). Calcium is important as it plays a role in maintaining shell thickness of *Pomacea* and preventing shell erosion (Glass & Darby 2009; Kwong et al., 2008).

We surveyed a map of Selangor to identify eight locations in different regions of the state that had wetlands or rice fields. Sampling was conducted from Sep 15th to Nov 7th 2016 from 9am - 3pm. Sample sites at these locations consisted of ponds, lakes, rice fields, or roadside ditches. At each locality, we surveyed one to six sites that were spatially separated by at least 30 m (i.e., different ponds/lakes/irrigation systems) so that a variety of mesohabitats were sampled. Three water samples were collected at each site, concentrations of selected electrolytes were measured using the LAQUAtwin water probes (HORIBA Instruments Inc, U.S.A), and the mean value was used for subsequent analyses. We collected 5–10 adult *Pomacea* snails at different sites for subsequent species identification using genetic analysis.

We conducted counts of *Pomacea* snails (>2 cm shell height) and egg masses at selected locations to obtain an index of relative abundance among sites. *Pomacea maculata* and *P. canaliculata* exhibit high interspecific similarity and intraspecific variability in shell and external morphology (Hayes et al., 2012); thus, we did not attempt to distinguish between species of *Pomacea* during counts. Counts were conducted along four 10 × 5 m transects selected at random points along the shoreline at each sampling site. Two individuals performed independent counts of abundance and the average of those counts were reported. Shells of collected specimens were cleaned, photographed and deposited in a reference collection at Sunway University.

Data Analysis

(i) Statistical Analysis

We explored associations between individual aquatic variables and snail counts (and egg mass counts) using Spearman rank correlation tests. We used binary logistic regression (Hosmer & Lemeshow, 1989) to assess the relationship between aquatic parameters and *Pomacea* presence/absence at 25 sample sites. We developed a set of eight *a priori* models that included one or more aquatic variables that we considered important predictors of *Pomacea* presence or absence and used Akaike's Information Criterion corrected for small sample sizes (AIC_c) to select the models best supported by the data (Burnham & Anderson, 2002). Because sample sizes were small, we limited the number of predictor variables to <2 in any single model and did not include interaction terms. Analyses were performed in Program R (v.3.3.1; R Development Core Team 2007).

(ii) Genomic Analysis

Genomic DNA was extracted from approximately 1 to 5 mg of foot tissue of individual snails using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany). A portion of the mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified and sequenced using the primers and thermocycle protocol published by Cooke et al. (2012). The PCR products were visualized in a 1% agarose gel before sequencing by MyTACG Biosciences Enterprise. Samples were sequenced using the BigDye® Terminator v1.1, v3.0 and v3.1 Sequencing Kit and analysed with Applied Biosystems 3730xl DNA Analyser. Sequencing data were analysed and edited using ChromasPro version 1.42 (2003-2008 Technelysium Pty Ltd) and BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) software. Edited sequences were aligned using CLUSTAL X alignment (Thompson et al., 1997) and visually checked before conducting phylogenetic analyses.

Sequences for the COI gene of *Pomacea* were downloaded from the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI). The generated partial COI mt DNA gene of *Pomacea* from selected localities together with the COI sequences downloaded from GenBank were used for the phylogenetic analysis. Maximum likelihood analysis was performed using Treefinder version October 2008 (Jobb et al., 2004) to construct phylogenetic trees (Swofford, 2002). Kakusan version 3 (Tanabe, 2007) was used to find the models with the best fit. We used Treefinder to build a phylogram of maximum likelihood using 1000 bootstrap replicates.

Results

Pomacea occurred in 5 of the 8 localities sampled and at 15 of 25 surveyed sites (Figure 1). Genomic analyses of 38 snails revealed two species of *Pomacea*: *P. canaliculata* and *P. maculata*. *Pomacea canaliculata* occurred in 5 of the 8 localities sampled (Figure 1) and co-occurred with *P. maculata* at two localities (Semenyih and Putrajaya). Fifteen sequences of *P. maculata (*synonymous with *P. insularum)*, *P. lineata*, and *P. canaliculata* were obtained from GenBank (Table 1). *Pomacea scalaris* and *P. diffusa* were used as outgroups in this study (Figure 1). *Pomacea canaliculata* was the more abundant and widespread of the two species, whereas *P. maculata* was documented only at Putrajaya and Semenyih, sympatric with *P. canaliculata.* The maximum likelihood tree produced 3 clades (A, B and C). *Pomacea canaliculata* from Putrajaya and Subang Jaya grouped with *P. canaliculata* sequences from Uruguay (Figure 1, Clade A) with 74% bootstrap support. *Pomacea canaliculata* from Sekinchan and Paya Indah clustered with *P. canaliculata* sequences from Argentina and Okinawa, Japan (Figure 1, Clade B) with 85% bootstrap support. *Pomacea maculata* sequences from Semenyih and Putrajaya clustered with *P. insularum* sequences from Okinawa, Japan; USA and Brazil (Figure 1, Clade C) with 98% bootstrap support. Sequences for the specimens used in this study were uploaded to GenBank.

Variation in depth, turbidity and vegetation at sample sites sometimes affected visibility and reliable snail counts. Counts of snails and egg masses ranged from 2–27 snails (averaged for two observers) and 5–50 egg masses, but

Table 1. GenBank Accession numbers for *Pomacea* species from different geographic locations.

Species	Location	GenBank Accession Number
P. maculata	Mato Grosso do Sul, Brazil	EU528559
P. maculata	Mato Grosso, Brazil	EU528568
P. maculata	Okinawa, Japan	AB433781
P. maculata	USA	JX845573
P. lineata	Algoas, Brazil	FJ710309
P. lineata	Rio de Janeiro, Brazil	FJ710310
P. lineata	Rio de Janeiro, Brazil	FJ710311
P. canaliculata	Maldonado, Uruguay	FJ710313
P. canaliculata	Buenos Aires, Argentina	EU528529
P. canaliculata	La Leonesa, Argentina	FJ710314
P. canaliculata	Buenos Aires, Argentina	FJ710315
P. canaliculata	Okinaw, Japan	AB433773
P. canaliculata	Kamimi, Japan	AB433767
P. scalaris	Buenos Aires, Argentina	EU528506
P. diffusa	Amazonas, Brazil	FJ710317

egg mass counts were only slightly correlated with snail counts (Figure 2; N = 15, Spearman's rho = 0.50, p = 0.08). Except for Semenyih, all sites with *Pomacea* had greater mean counts of egg masses than mean counts of snails (Figure 2). Variation was high among counts taken along different transects at the same wetland site, ranging from 8 to 30 snails and 5–53 egg masses. Consequently, snail counts and egg mass counts were not correlated with any aquatic parameter. Thus, we used presence/absence of *Pomacea* to investigate further relationships. The highest values for all measured parameters were from Sepang and Kuala Selangor (Figure 3). *Pomacea* were absent at these two sites, including Jing An, where water parameters were not substantially different from other sample sites. Marked differences in aquatic parameters existed between sites with and without *Pomacea*, sites without *Pomacea* had higher pH and electrolyte concentrations (Figure 4).

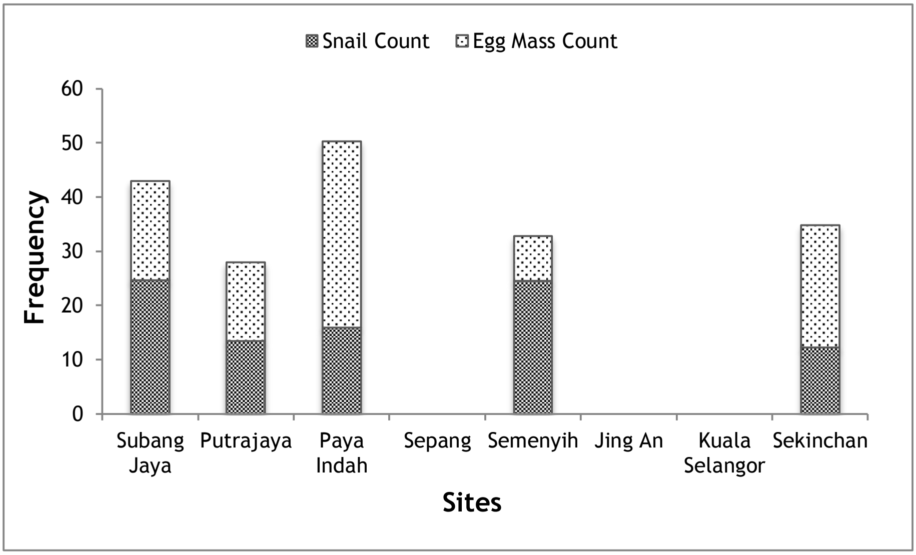

Figure 2. Mean counts of *Pomacea* snails and egg masses observed at eight different sites in Selangor, Peninsular Malaysia from September to November 2016.

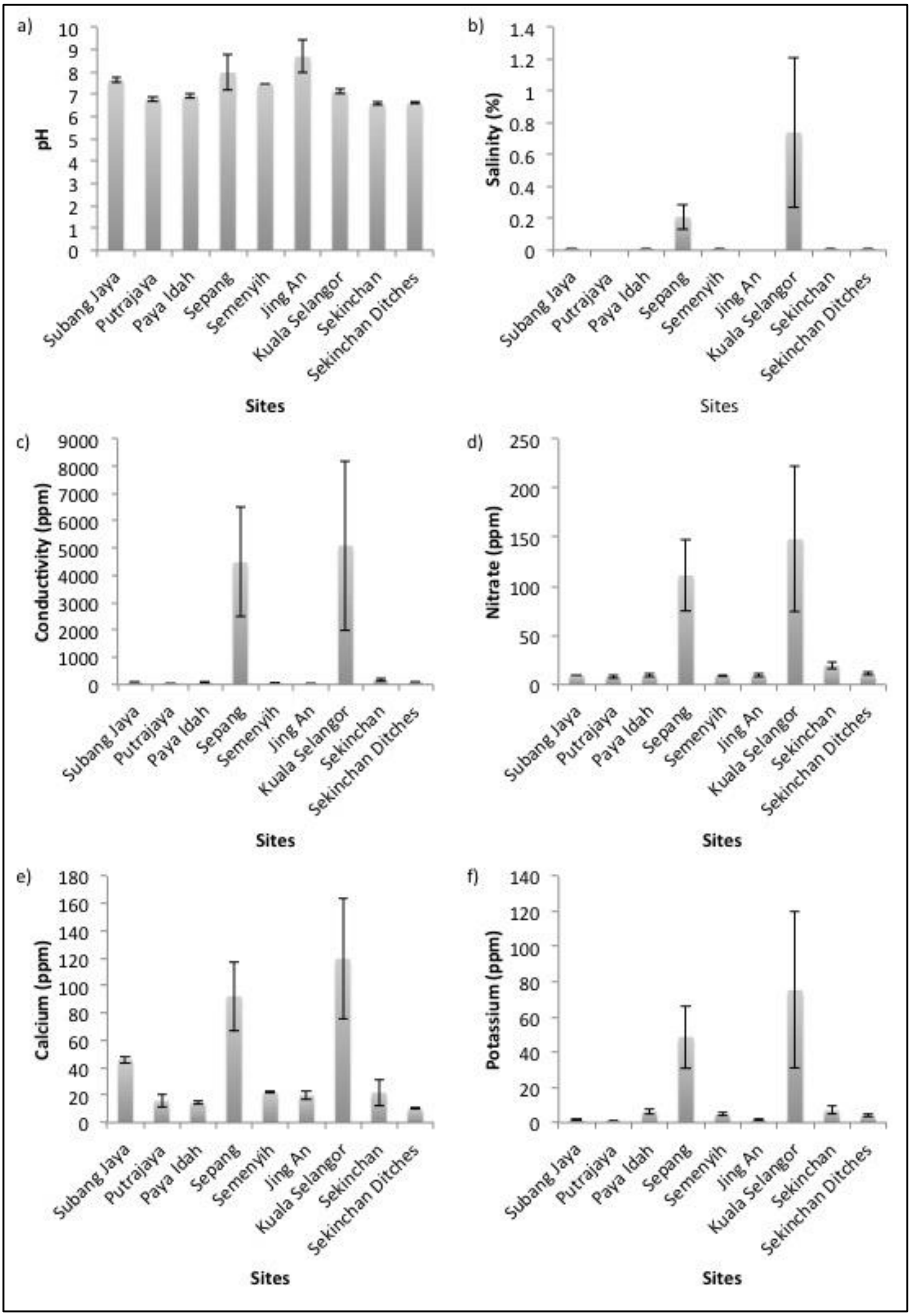

Figure 3. Comparison of means of different aquatic parameters among sampling localities in Selangor. Mean values for roadside ditches at Sekinchan are reported separately from those collected from rice fields. Error bars represent standard errors of the mean.

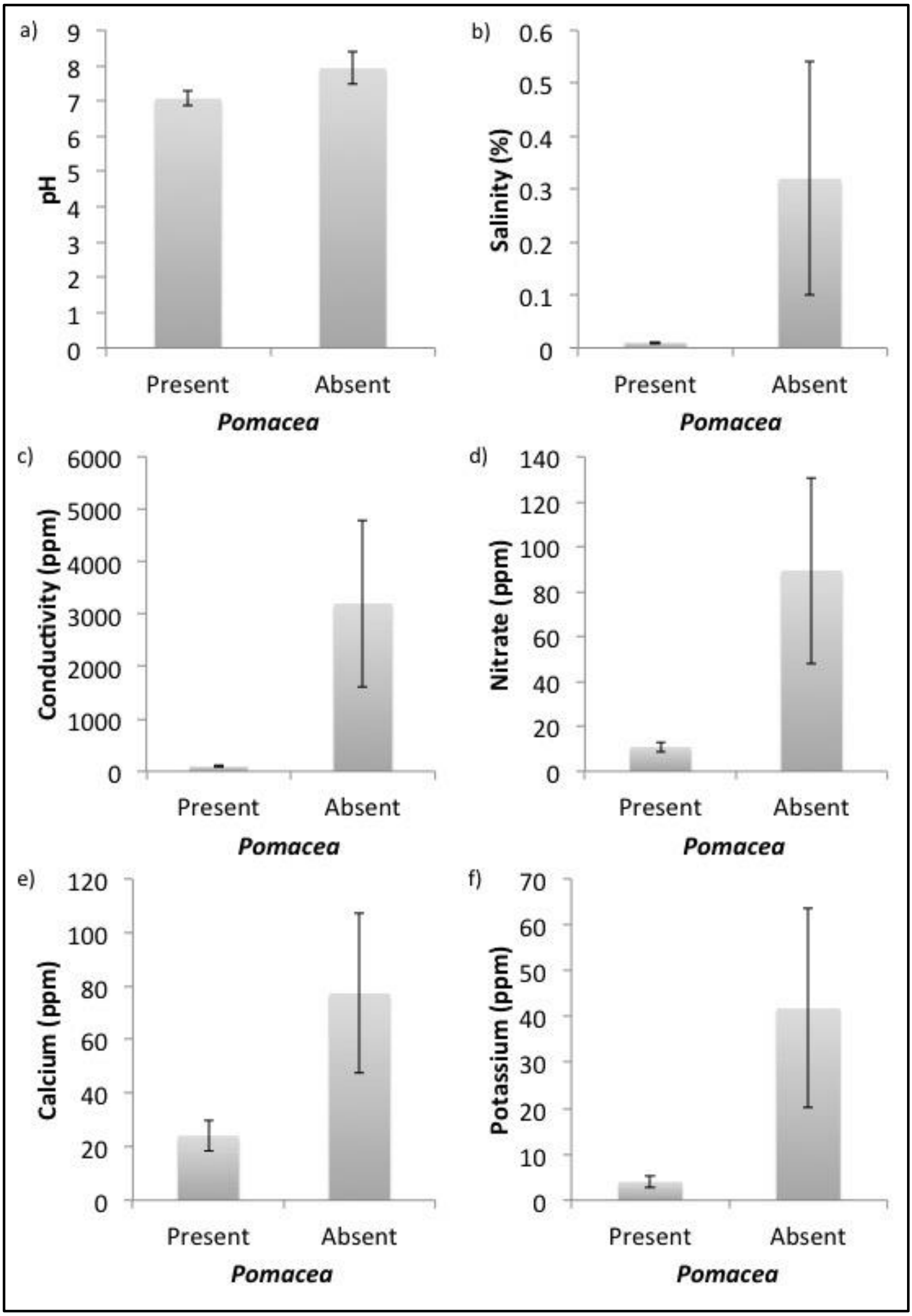

Figure 4. Relationship between the overall means of different aquatic parameters across all localities and presence/absence of *Pomacea*. Error bars represent standard errors of the mean.

We developed 8 logistic regression models representing the association between one or a combination of two aquatic parameters and presence or absence of *Pomacea* (Table 2). The most supported model was the combined model of pH and calcium concentration (lowest AIC_C value and a ΔAIC_C >2 compared to the next ranked model; Table 2). The parameter estimates for pH and calcium were negative, although not significant for calcium (Table 3). Data indicated that *Pomacea* occurred at sites with lower pH (6.5 – 7.5) and lower calcium ion concentrations (16-46 ppm). Sites with high pH (>8) and calcium concentrations (> 90 ppm) were associated with *Pomacea* absence. *Pomacea* were absent at sites with the highest levels of all aquatic variables measured, although models with those variables had poor fit, possibly because the distribution of those variables were distinctly skewed (Figure 3). Calcium concentrations were strongly correlated with salinity (Spearman's rho = 0.73, p = 0.0001) and conductivity (Spearman's rho = 0.76, p = 0.0001); thus overall aquatic ion concentrations were high where calcium and salinity levels were high.

Table 2. AIC-based logistic regression model selection for the presence/absence of *Pomacea* evaluated against six different aquatic parameters. Akaike Information Criterion corrected for small sample size (AIC_c), AIC_c differences (ΔAIC_c), Akaike weights (w_i), and number of estimable parameters (K).

Model	AIC_c	ΔAIC_c	w_i	K
1. pH + calcium	24.420	0.000	0.510	3
2. Conductivity	26.841	2.421	0.152	2
3. Nitrate	27.721	3.301	0.098	2
4. Salinity	28.069	3.649	0.082	2
5. Potassium	28.327	3.907	0.072	2
6. pH	29.339	4.919	0.044	2
7. Potassium + nitrate	30.285	5.865	0.027	3
8. Calcium	31.433	7.013	0.015	2

Table 3. Coefficients of top ranking logistic regression model for parameters predicting *Pomacea* presence/absence. Calcium concentration and pH were negatively associated with snail presence.

	Estimate	Standard error	z value	Probability
Intercept	17.097	7.207	2.372	0.018
pH	-2.063	0.958	-2.152	0.031
Calcium	-0.037	0.019	-1.917	0.055

Discussion

Phylogenetic analysis confirmed the presence of the two most invasive species in the genus *Pomacea* in wetlands of Selangor. *Pomacea canaliculata* occurred in approximately five localities and *P. maculata* in two (Putrajaya and Semenyih) where it co-occurred with *P. canaliculata*. Genetically, the two species separated clearly with no shared haplotypes. *Pomacea canaliculata* occurred at 15 of the 25 sample sites and *P. maculata* at just four. Based on this limited survey, *P. canaliculata* seems the more widespread of the two species in Selangor.

The strongest predictors of *Pomacea* presence/absence were pH and calcium ion concentration where the probability of *Pomacea* presence was low when pH and calcium concentrations were high. All aquatic parameters were noticeably higher at sites in Sepang and Kuala Selangor where *Pomacea* was absent, suggesting that *Pomacea* may be physiologically limited by the total amount of dissolved salts in the water, which causes osmotic stress (Costil et al., 2001; Ramakrishnan, 2007). Under laboratory conditions, *P. bridgesi* and *P. maculata* tolerate salinities between 0 – 6.8% quite well (Ramakrishnan, 2007; Jordan & Deaton, 1999). In Hong Kong, freshwater locations inhabited by *P. canaliculata* had high alkalinity, high levels of phosphate and high salinity (Kwong et al., 2008; Chaichana & Sumpan, 2015). Furthermore, Rossi (2012) commented that salinity appears to not limit *Pomacea*'s establishment in an area. In this study, the highest salinities recorded at sites in Sepang and Kuala Selangor did not exceed 1.64%. Sites at Sepang were isolated from tidal influence, but sites at Kuala Selangor may experience spikes in salinity that *Pomacea* cannot tolerate. The absence of *P. canaliculata* and *P. maculata* in Sepang and Kuala Selangor may also mean that introduction to these sites or dispersal via canals and drainages has not yet occurred.

Both high and low aquatic pH influences aquatic biodiversity and productivity (Bemvenuti et al., 2003). *Pomacea* may be exceptional in its ability to tolerate aquatic pH as low as 4.5 in lab settings (Ramakrishnan, 2007), but whether survival and reproduction occurs over the long term under these conditions, is unknown. In temperate regions like Japan, with marked seasonal fluctuations in temperature, low water velocity, high dissolved oxygen and low pH (6.29-6.63) is associated with greater over-winter survivorship of *P. canaliculata* (Ito, 2002, 2003). Most sites surveyed in this study had pH levels ranging from 6.5–7. The possibility that low pH habitats such as peat wetlands may be vulnerable to invasion should be considered, although Byers et al. (2013) reported absence of *P. maculata* in southeastern U.S. waters with pH <5.5 and Pierre

(2015) reported decreasing survivorship of translocated *P. maculata* at aquatic pH <6. Generally, low pH affects the ability of gastropods to deposit calcium in their shells, and calcium plays a crucial role in maintaining shell thickness in gastropods and preventing shell erosion (Glass & Darby, 2009). The lowest calcium levels were recorded at Paya Indah and Putrajaya wetlands where *Pomacea* populations were abundant, but where pH levels were between 6.5-7.0. Lab experiments and a greater coverage of these parameters in Malaysian wetlands may help to elucidate the relationship between pH, calcium, and *Pomacea* presence/absence. This is the first report of aquatic parameters associated with the presence/absence of *P. canaliculata* and *P. maculata* in Malaysian wetlands. Similar kinds of information from different parts of its introduced range will help establish its full physiological range of tolerance and invasive potential.

Obtaining crude relative abundance estimates of *Pomacea* populations using egg mass counts needs to be further explored. Our data suggested that snail counts and egg mass counts were weakly correlated. *Pomacea*'s conspicuously pink coloured egg masses are deposited above the waterline on the stalks of emergent aquatic plants or inanimate structures, making accurate counts possible. Snail counts, on the other hand, may be hampered by turbid water conditions or cryptic shell coloration and pattern (Burks et al., 2010). We observed that snails were often hidden among aquatic plants, and were well camouflaged. Surveying for the presence of egg masses is an efficient and practical way to ascertain presence of the snail, but egg mass counts were only weakly correlated with snail counts. Seasonal patterns of egg deposition must be investigated against snail counts to use egg mass counts as an index of abundance.

The success of *Pomacea* as an invader owes largely to its ecological competence and high adaptability to a range of aquatic habitats and environmental conditions, including seasonally dry lands (Chaichana & Sumpan, 2015; Hayes et al., 2015; Glasheen et al., 2017). *Pomacea maculata* and *P. canaliculata* may be quite opportunistic, with a highly variable diet ranging from macrophytes to animal carcasses (Carlsson & Brönmark, 2006). Both *P. maculata* and *P. canaliculata* possess remarkable reproductive capacity and introduced populations expand rapidly at the expense of native species. In Southeast Asia, *Pomacea canaliculata* reproduces three times faster under the warm climate as compared to its native cold and seasonal South American habitat (Carlsson & Lacoursiere, 2005). Snails in the genus *Pomacea* may be capable of surviving harsh environments including pollution, low oxygen levels, or lack of water, because ampullariids are equipped with lungs and gills to accommodate aerial and aquatic breathing (Baloch et al., 2012; Hayes et al., 2015). Both *P. canaliculata* and *P. maculata* possess a remarkably flexible operculum that acts as a trapdoor to tightly seal the snail from the exterior, thus protecting it from desiccation for months at a time (Kwong et al., 2009). In combination, these characteristics have contributed to the spread and destructiveness of these two species on a global scale.

Conclusion

Two of the most invasive species in the genus *Pomacea* occur in Selangor. We did not include peat swamp habitats in this study and preliminary surveys have not detected *Pomacea* in the Northern Selangor peat swamp, which is upstream and adjacent to Sekinchan where *Pomacea* occurs. *Pomacea*'s reported tolerance for low pH suggests that peat swamp habitats may be vulnerable to invasion and should be monitored. Osmotic stress may limit *Pomacea*'s ability to invade brackish environments such as alkaline lakes and mangrove habitats. *Pomacea canaliculata* and *P. maculata* are capable of dispersing large distances, drifting along currents in natural and artificial drainages, and *Pomacea* eggs and hatchlings may be transported from one region to another via aquatic plants used for landscaping and aquaria (Pierre, 2015; Ng et al., 2017). It is important that public awareness via educational brochures, digital media and magazine/journal articles is increased so that people have information to 1) identify invasive apple snails, 2) recognize activities that can inadvertently contribute to the spread of invasive snails, and 3) use environmentally safe ways to control their spread and reproduction.

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