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Differences in reproductive effort and sexual recruitment of the seagrass *Zostera japonica* between two geographic populations in northern China

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ABSTRACT: Coastal seagrass beds are pivotal but threatened marine ecosystems throughout the world. The seagrass *Zostera japonica* Asch. & Graebn. is an endangered species in its native range along the northwestern Pacific coast. In this study, we used ecological survey methods and microsatellite analysis to evaluate sexual reproduction and its role in recruitment of *Z. japonica* populations at Swan Lake lagoon (SLL) and Huiquan Bay (HQB) in northern China. Mixed annual and continuous meadows of *Z. japonica* at SLL produced a high number of seeds (mean ± SD: 40 244 ± 18 666 seeds m^{-2}) and formed a relatively stable seed bank (1460 ± 417 seeds m^{-2}) in the sediment. About 41% of the seed bank and 6% of shoots survived over winter, and recruitment from seeds accounted for 41 ± 24%. In contrast, perennial and fragmented *Z. japonica* at HQB had lower seed production (12 501 ± 5748 seeds m^{-2}) and a much smaller seed bank (10 ± 6 seeds m^{-2}). About 66% of shoots survived over winter, but seedling recruitment was rare at HQB. Thus, relatively large differences in genetic and clonal diversity were predicted between SLL and HQB. Results of the microsatellite analysis of samples collected in 2012 and 2015 showed higher clonal (R) and genetic diversity (H_o) at SLL (2015: R = 1; H_o = 0.55) than at HQB (2015: R = 0.40; H_o = 0.42). These results highlight the role of sexual and asexual reproduction in maintenance and evolutionary connectivity of seagrass populations and emphasize the need to understand local recruitment strategies before starting restoration and management projects.

KEY WORDS: Endangered species · Sexual reproduction · Seedling recruitment · Seed bank · Clonal growth · Clonal diversity

1. INTRODUCTION

All seagrass species colonize the sea through sexual (via seeds) and asexual (via clonal growth of rhizomes) reproduction. Early studies considered asexual reproduction to be the primary process responsible for the maintenance and establishment of seagrass meadows (e.g. Procaccini & Mazzella 1998, Rasheed 2004), because successful recruitment via sexual reproduction is limited due to low pollination success, restricted dispersal of pollen and seeds, and low survival of seeds and seedlings (Les 1988, Laush-

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man 1993, Reusch 2003). However, a number of more recent studies based on genetic tools and/or ecological observations contradict the previous perception that sexual reproduction is not important for seagrasses (Kendrick et al. 2012, S. C. Xu et al. 2018). First, extensive seedling recruitment (e.g. Balestri & Lardicci 2008, Zipperle et al. 2009a, Jarvis & Moore 2010, Smith et al. 2016) and long-distance dispersal of sexual propagules (Kendrick et al. 2012) have been observed for different seagrass species. Moreover, high outcrossing rates and multiple paternities were found in *Zostera marina* (Reusch 2000), *Z. noltii* (Zipperle et al. 2011), and *Z. muelleri* (Sherman et al. 2016), indicating that pollination success is high. Even in persistent species such as *Posidonia australis*, outcrossing is obligate with multiple paternities, and median successful pollination occurs over tens of meters, with extreme pollination distances >100 m (Sinclair et al. 2014). In addition, reports of high genetic diversity in different seagrass species (Procaccini & Mazzella 1998, Reusch et al. 1999, Coyer et al. 2004, Hernawan et al. 2017) indicate that the role of sexual reproduction may have been underestimated previously.

Successful sexual recruitment is generally constrained by bottlenecks in the reproductive cycle of seagrasses, particularly in the key steps of flowering, fruiting, seed production, germination, and seedling development (Kendrick et al. 2017). Reproductive efforts (timing, period, flowering shoot density, and ratio of flowering to total shoots) (Alexandre et al. 2006, Park et al. 2011) and sediment seed banks (size, viability, temporal and spatial patterns) (Inglis 2000, Harwell & Orth 2002, Zipperle et al. 2009a, Jarvis et al. 2014) have been studied intensively, especially in model species such as *Z. marina*, whereas the transition from seeds to seedlings may be the most crucial, but also the most poorly understood stage (Marion & Orth 2012). Salinity (Fernandez-Torquemada & Sanchez-Lizaso 2013), temperature (Xu et al. 2016), seed age (Kaldy et al. 2015), light (Bintz & Nixon 2001, Abe et al. 2010), sediment nutrients (Statton et al. 2014), burial depths (Cumming et al. 2017), microsites (van Katwijk & Wijgergangs 2004, Rivers et al. 2011, Alagna et al. 2013), and predation (Zipperle et al. 2010, Manley et al. 2015) all may influence this transition. However, sexual recruitment dynamics and the contribution of sexual reproduction to natural populations have not yet been quantified, which affects our ability to predict the importance of sexual reproduction for population maintenance and regulation (Kendrick et al. 2017).

Z. japonica Asch. & Graebn. is an intertidal species that is native to the Western Pacific Ocean from Russia to Vietnam (Miki 1933) and was introduced into North America in the 1950s (Harrison & Bigley 1982), where it spread quickly along estuaries during the past 3 to 5 decades (Shafer et al. 2014). In contrast, this species is widely threatened in its native range in Korea, Japan, and China (Lee 1997, Lee et al. 2004, Abe et al. 2009, Zhang et al. 2019).

Z. japonica is among the least studied seagrass species, and there are many uncertainties about the role of sexual reproduction and population recruitment in maintaining populations. Only a few studies have focused on the temporal and spatial patterns of sexual reproduction in natural *Z. japonica* populations under different disturbance conditions or exposure zones (Henderson & Hacker 2015, Suonan et al. 2017, Zhang et al. 2019). Several studies used controlled experiments to examine the effects of different factors (temperature, salinity, seed age, tidal elevation, and sediment type) on seed storage, germination, and/or seedling establishment. Temperature and salinity were reported to have a strong influence on seed storage and germination. For example, low temperature (0–7°C) and high salinity (40–60 psu) were required for seed storage (Kishima et al. 2011, Morita et al. 2011, Yue et al. 2019a), and optimal temperature for seed germination ranged from 15 to 30°C depending on geographic origin (Abe et al. 2009, Yue et al. 2019b). Salinity had much stronger control over seed germination compared to temperature; low salinity (<10 psu) stimulated germination (Kaldy et al. 2015, Yue et al. 2019b), but seedling establishment required higher salinity (>10 psu; Yue et al. 2019b) and temperatures below 29°C (Abe et al. 2009). In addition, a higher seed germination rate was reported for seeds that were stored for a longer time (Kaldy et al. 2015).

Understanding recruitment is essential to the conservation and management of seagrasses, and this study was conducted to better understand the recruitment processes of *Z. japonica* populations in different habitats and with different growth forms. We studied 2 populations from northern China: the first was a continuous mixed-annual meadow at Swan Lake lagoon, and the other was a patchy perennial meadow at Huiquan Bay. The goals of this study were to (1) compare the temporal and spatial patterns of biomass, flowering efforts, seed production, seed bank density, and seedling recruitment; (2) assess differences in genetic and clonal diversity; and (3) compare the contribution of sexual reproduction to population recruitment between the 2 populations.

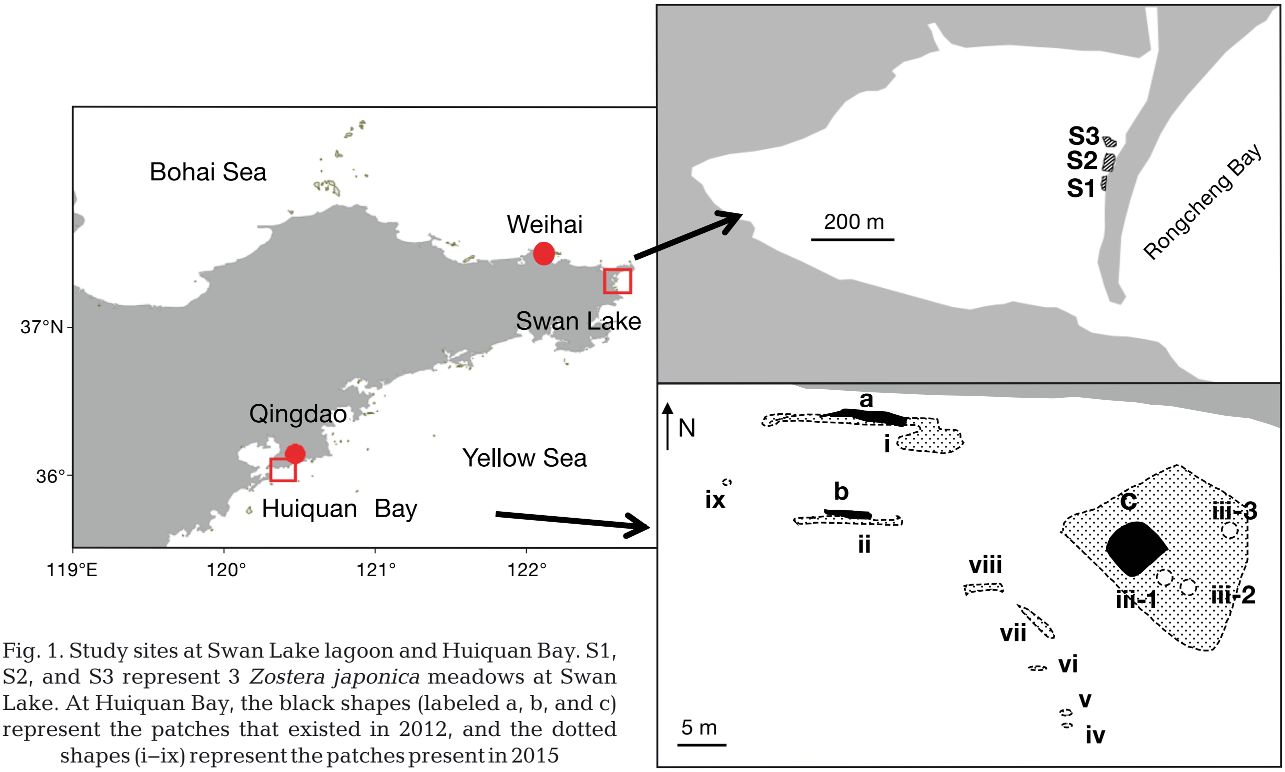

Fig. 1. Study sites at Swan Lake lagoon and Huiquan Bay. S1, S2, and S3 represent 3 *Zostera japonica* meadows at Swan Lake. At Huiquan Bay, the black shapes (labeled a, b, and c) represent the patches that existed in 2012, and the dotted shapes (i–ix) represent the patches present in 2015

2. MATERIALS AND METHODS

2.1. Study sites

Swan Lake lagoon (SLL; 122° 34′ E, 37° 21′ N) is located in Weihai, northern China (Fig. 1). SLL is a marine lagoon that has an area of 4.8 km^2 and is connected to the Yellow Sea by a narrow inlet with a width of 86 m. There are irregular semidiurnal mixed tides (tidal range = 1.65 m). The water temperature varies seasonally from −1.3 to 25.6°C. The lagoon is very shallow and functions as a suitable habitat for the seagrasses *Zostera marina* and *Z. japonica* (Zhang et al. 2014, 2015a, Zhou et al. 2015, Q. Z. Xu et al. 2018, S. Xu et al. 2019). *Z. japonica* mainly occurs in the narrow mid-upper intertidal zone (Zhang et al. 2015a). Three plots (S1– S3, each 15 m × 15 m; Fig. 1) were established along the coast to investigate the temporal and spatial pattern of sexual reproduction of *Z. japonica*.

Huiquan Bay (HQB; 120° 34′ E, 36° 05′ N; Fig. 1) is located in Qingdao, northern China (Zhou et al. 2014). HQB is open to the southwest where it faces the Yellow Sea. It has regular semidiurnal tides (tidal range = 4.8 m). A meadow with mixed patches of *Z. marina* and *Z. japonica* is distributed in the intertidal zone in the southeastern corner of the bay. Only 3 *Z. japonica* patches (a, b, and c; Fig. 1) were present in 2010–2012, but 6 new patches (iv–ix) appeared during 2014–2015 with areas of <1 m^2 to tens of m^2; moreover, the areas of patches a, b, and c had all increased, with the largest patch ca. 250 m^2. Considering that most patches of *Z. japonica* were relatively small, we regarded the whole meadow as a single plot for the field investigation.

2.2. Environmental parameters

Water temperatures (°C) at SLL and HQB were measured every 15 min from March 2014 to December 2015 using a HOBO Pendant light/temp UA 00-64 (Onset). Salinity was measured monthly with a Pro30 Conductivity, Salinity Instrument (YSI). Light intensity at the canopy was captured by an ECO-PARSB sensor (Sea-Bird Scientific) deployed in the centers of the *Z. japonica* meadows at SLL and HQB from January/February to December 2015. Instantaneous photosynthetic photon flux densities (PPFDs; mol photons m^{-2} s^{-1}) were measured every 10 min, and daily PPFDs (mol photons m^{-2} d^{-1}) were calculated as the sum of the quantum flux within a 24 h period. Three sediment cores (diameter = 10.6 cm, height = 12 cm) were collected in each plot for determination of grain size distribution. Sediments in each core were homogenized, and subsamples were then analyzed using sequential sieving (Erftemeijer & Koch 2001) and/or laser particle size analysis using a particle size analyzer (CILAS 1190L).

2.3. Clonal growth of adult shoots

From March 2014 to December 2015, the growth changes in shoot density, shoot height, and biomass of adult shoots at SLL and HQB were investigated once each in summer (July or August), autumn (October), and winter (December) and multiple times during spring to early summer (March to June) to observe seed germination and seedling growth. Two surveys at SLL in spring to early summer were conducted (i.e. before and after the seed germination period), and overwintering shoots and seedlings recruited via seeds were sampled. Because it was difficult to determine the seed germination period at HQB, multiple samplings were conducted there to distinguish seedlings from adult shoots.

Six sediment cores (diameter = 15.4 cm, height = 12 cm) were collected randomly within each of the 3 plots at SLL, and 3 to 6 cores were collected within each of the 3 largest patches at HQB. All samples were sieved (2 mm) with seawater *in situ* to remove most of the sediment, and the plant materials were taken to the laboratory and cleaned using tap water. For each sample, total number of shoots (including flowering and vegetative shoots) was counted to provide shoot density (shoots m^{-2}). Next, 20–30 shoots were randomly chosen for measurement of shoot height (distance from the bottom of the sheath to the top of the longest leaf) and individual fresh weight (FW) biomass (above- and belowground parts separately). Individual shoot biomass was used to calculate total biomass per unit area (g FW m^{-2}).

2.4. Flowering and seed production

During the flowering periods in 2014 and 2015, flowering shoots and their seed production at SLL and HQB were investigated based on the same cores described in the previous section. For each core, we counted the numbers of flowering and vegetative shoots and the flowering shoot density (shoots m^{-2}), and the ratio of flowering shoots to total shoots (%) was then calculated. Additionally, 20–30 intact flowering shoots in each sampling were randomly chosen to quantify the number of spathes per flowering shoot, pollinated spathes per flowering shoot, and number of seeds per spathe. The potential seed production per shoot (seeds shoot^{-1}) was calculated by multiplying the seeds per spathe by the total number of spathes per flowering shoot. Potential seed production per unit area (seeds m^{-2}) was estimated by multiplying the maximum of seed production per shoot with the maximum density of flowering shoots during the flowering period.

2.5. Seed banks

Surveys of seed banks at SLL were conducted monthly from late October 2014 to mid-November 2015. Initially, 7 sediment cores (diameter = 15.4 cm, height = 12 cm) were randomly collected in each plot, but we increased the number of cores in each plot to 10 from April to June 2015 when seed bank density decreased dramatically.

Surveys of seed banks at HQB started in early October 2014, and a total of 6 surveys (October and December 2014; March, early and late October, and December 2015) were conducted for practical reasons. Sediment cores (n = 30–33) were randomly collected within and outside *Z. japonica* patches, including within the *Z. marina* meadows and in bare areas. A total of 7–10 cores were collected in the largest patch (c), and 2 cores were collected in each of patches a and b. Outside the patches, 19 cores were collected randomly, and the distance of each core from the nearest patch was recorded.

The sediment cores were homogenized and sieved twice using mesh sizes of 0.7 and 1.5 mm. Seeds mixed with fine sands and detritus were retained in the 0.7 mm mesh, while larger plant parts or detritus were retained in the 1.5 mm mesh. The mixture of seeds and fine particles was stored at 4°C until processing, which was conducted within 1 wk. The seeds and seed coats were collected from the sample mixtures and pressed using tweezers. The rotten seeds and seed coats were flat (S. C. Xu et al. 2018). The intact seeds were counted to calculate seed bank density (seeds m^{-2}).

2.6. Seedling recruitment at SLL

At SLL, a permanent quadrat survey was conducted in spring 2014 to trace seed germination, and a random sampling survey was carried out in spring 2015 to quantify the contribution of seedling recruitment. Seedlings were very rare at HQB, thus, seedling recruitment was not investigated at that site.

In the permanent quadrat survey, a total of 10 permanent quadrats (18.5 × 18.5 cm) were established to track changes in the numbers of seedlings and overwintering shoots from early March to June 2014. Seedlings and overwintering shoots within the quadrats were counted biweekly at low tide. Newly emerged seedlings were easily recognized and distinguished from the larger, overwintering shoots based on their thin white cotyledon and small green leaves at the beginning of the survey. In rare cases, we also verified them by excavating the whole individual in order to expose the rhizomes and/or the original seed coat, which mainly happened in the later stage of this survey.

In the random sampling survey, 6 cores (size = 30 × 30 cm, depth = 12 cm) with sediment, seedlings and overwintering shoots were sampled from March to June 2015. The samples were sieved (1.5 mm) carefully *in situ*, and the retained seagrass materials, including seedlings and overwintering shoots, were taken back to the lab for further processing. The numbers of seedlings, shoots per seedling, total seedling shoots, and overwintering shoots were counted.

2.7. Microsatellite analysis

To study the recruitment strategy (clonal versus sexual reproduction) of *Z. japonica* at HQB, the genetic (allelic) and clonal (genotypic) diversity of the patches in HQB were examined based on 2 groups of samples collected in 2012 and 2015, respectively. In 2012, a total of 34 shoot samples were haphazardly collected at intervals of 2–3 m within all 3 patches, while 84 samples were collected in 2015 separated by 0.15–1 m from 9 existing patches. The distances between samples in 2015 were adjusted according to the size of each patch to make sure that at least 3 samples were collected in each patch. For each sample (genet) consisting of 2 or 3 shoots (ramets) connected by a rhizome, the inner fresh leaves and sheaths were cleaned with deionized water and then stored at −80°C. For comparison, 40 samples were collected haphazardly with an interval of 2–10 m in 2012 in SLL and 24 samples were collected in 2015 at an interval of 1 m within an area of 5 m × 5 m located in the center of plot S2. Seven polymorphic microsatellite loci were amplified by PCR with the published primers Zj 008, 028, 025, 042, 026, 018, and 011 (Zhang et al. 2015b). The DNA extraction and PCR amplification procedures were described by Zhang et al. (2015b).

2.8. Data analysis

Values are represented as means ± SD. Differences in temperature and light intensity between sites were analyzed using repeated measures ANOVA. Differences in sediment grain proportions between sites were tested with non-parametric statistics. The effects of sites on the maximum and minimum values of shoot height, biomass, and belowground biomass proportions in winter and summer and on flowering shoot proportions in the reproductive period were tested using 2-way ANOVA. Differences in the minimum and maximum values of shoot density, flowering shoot density, spathes per flowering shoot, seeds per spathe in their most reproductive period, and seed bank density between sites were separately analyzed using a generalized linear model (GLM-Poisson regression) with time and site as factors. Temporal changes in density of seedlings and overwintering shoots in the random survey were also analyzed using the GLM-Poisson regression. Differences in the number of seedling shoots in the permanent quadrat survey were tested using repeated measures ANOVA (n = 7). Prior to analysis, data were transformed (square root or arcsin-square root) when necessary. All analyses were conducted using SPSS 23.0. Differences were considered significant at $p < 0.05$.

Two or more ramets might share the same multilocus genotype (MLG) due to the sampling of multiple shoots from a single clone (genet), or by the chance recombination of identical alleles during sexual reproduction. P_{sex}, the probability that 2 or more identical genotypes arose due to sexual reproduction, was calculated using Genclone 2.0 (Arnaud-Haond & Belkhir 2007) for ramets that shared the same MLGs, and if $P_{sex} < 0.01$, this indicated that the identical ramets came from the same clone, and thus the duplicate MLGs were considered only once in the subsequent analysis. Clonal or genotypic diversity R was estimated as: $R = (G - 1) / (N - 1)$, where G is the number of MLGs (genets) and N is the number of ramets sampled. Measures of genetic diversity comprising the observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC), and the mean number of alleles per locus (N_a) were calculated using the Excel Microsatellite Toolkit (Park 2001).

3. RESULTS

3.1. Environmental parameters

Water temperature averaged 15.8 ± 7.5°C at SLL and 17.5 ± 7.5°C at HQB (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m638p065_supp.pdf). Winter was relatively colder at SLL, and the water surface was ice-covered on the coldest days. By comparison, winter was warmer at HQB with an extremely low probability of snow and ice. Summer was also cooler at SLL (23.7 ± 1.5°C) than at HQB (26.6 ± 1.4°C). The PPFD in HQB varied with a larger range than that of SLL (0.7~56.4 vs. 1.7~38.0 mol photons m^{-2} d^{-1}), but the annual averages were very similar (15.5 vs. 15.0 mol photons m^{-2} d^{-1}; Fig. S2). Salinities at SLL and HQB were similar, with annual averages of 30.8 ± 2.2 and 30.5 ± 2.0 psu, respectively. The sediments at SLL and HQB both were mainly composed of sands and silt (Table S1). The proportion of sands was lower at SLL than at HQB ($p < 0.05$), while the content of silt was similar at the 2 sites ($p > 0.05$).

3.2. Temporal changes in density, height, and biomass of adult shoots

Shoot density, shoot height, and (above- and below-ground) biomass of adult shoots varied seasonally at both sites (Fig. 2A,B; $p < 0.001$), with peaks in summer and minimum values in winter and early spring. Shoot density, shoot height, and total/below-ground biomass were generally higher at HQB than at SLL ($p < 0.05$), although maximum values of these parameters did not differ between sites ($p > 0.05$).

3.3. Flowering and seed production

Duration of the flowering period (Fig. 3A) varied between years at SLL and lasted for 5 mo in 2014 (June to October) and 6 mo in 2015 (June to December). The flowering period in HQB was much shorter, lasting for 3–4 mo, from June to September (Fig. 3B).

Zostera japonica at SLL mainly flowered within 3 mo, from July to September 2014 and August to October 2015. The flowering shoot density and proportion at SLL were highest in August 2015 and in September 2014 (1767 ± 819 shoots m^{-2} and 37.5 ± 8.4%, respectively). However, *Z. japonica* at HQB mainly flowered between July and August in both 2014 and 2015, and the flowering shoot density and proportion in 2014 were both lower than in 2015 ($p < 0.05$). The highest density of flowering shoots (1161 ± 986 shoots m^{-2} in July 2015) and the highest proportion of flowering shoots (32.9 ± 16.7% in August 2015) at HQB were similar to those at SLL ($p > 0.05$).

In 2015, the number of spathes per flowering shoot increased with time at SLL and reached a maximum of 5 ± 2 at the end of August. Formation of seeds was first observed in mid-July, and most seeds matured from August to October (Fig. 4A). The number of seeds per spathe was highest in late August (5 ± 1, with a mode of 6). The largest potential seed production per flowering shoot was 23 ± 8 in August, and the potential seed production per unit area in 2015 was 40 244 ± 18 666 seeds m^{-2}.

Seed formation at HQB began a month earlier than at SLL (Fig. 4B). The number of spathes per flowering shoot peaked in late August (4 ± 3) and the maximum number of seeds per spathe peaked in July (4 ± 1), but both values were lower than those at SLL ($p < 0.05$, $p < 0.01$, respectively). At the same time, the number of seeds per spathe was 6 at most, with a mode of 2. The potential seed production per flowering shoot was 12 ± 8, which was lower than that at SLL ($p < 0.05$). The potential seed production per unit area at HQB was 12 501 ± 5748 seeds m^{-2} in 2015, which was lower than that at SLL ($p < 0.05$).

3.4. Seed bank

At SLL, the seed bank density in the sediment exhibited a clear temporal pattern ($p < 0.01$). The highest seed bank density (1460 ± 417 seeds m^{-2}) occurred in October 2014, but it declined quickly beginning in January 2015 to reach a low number in June 2015 (6 ± 3 seeds m^{-2}), and then a new seed bank was formed by the end of the flowering season (1334 ± 63 seeds m^{-2} in November). The maximum seed density in 2015 accounted for ca. 3% of the potential seed production. There were no significant differences in the seed density between years and among plots ($p > 0.05$).

At HQB, no seeds were found in most surveys except the one conducted in early October 2015 (Fig. 5B). At that time, the seed density was 10 ± 6 seeds m^{-2}, accounting for <0.1% of the potential seed production. Only 3 of the 10 cores were located in the *Z. japonica* patches, with 4 cores located in the *Z. marina* meadow and 3 in the bare areas. The maximum distance that seeds were found outside of *Z. japonica* patches was 15 m.

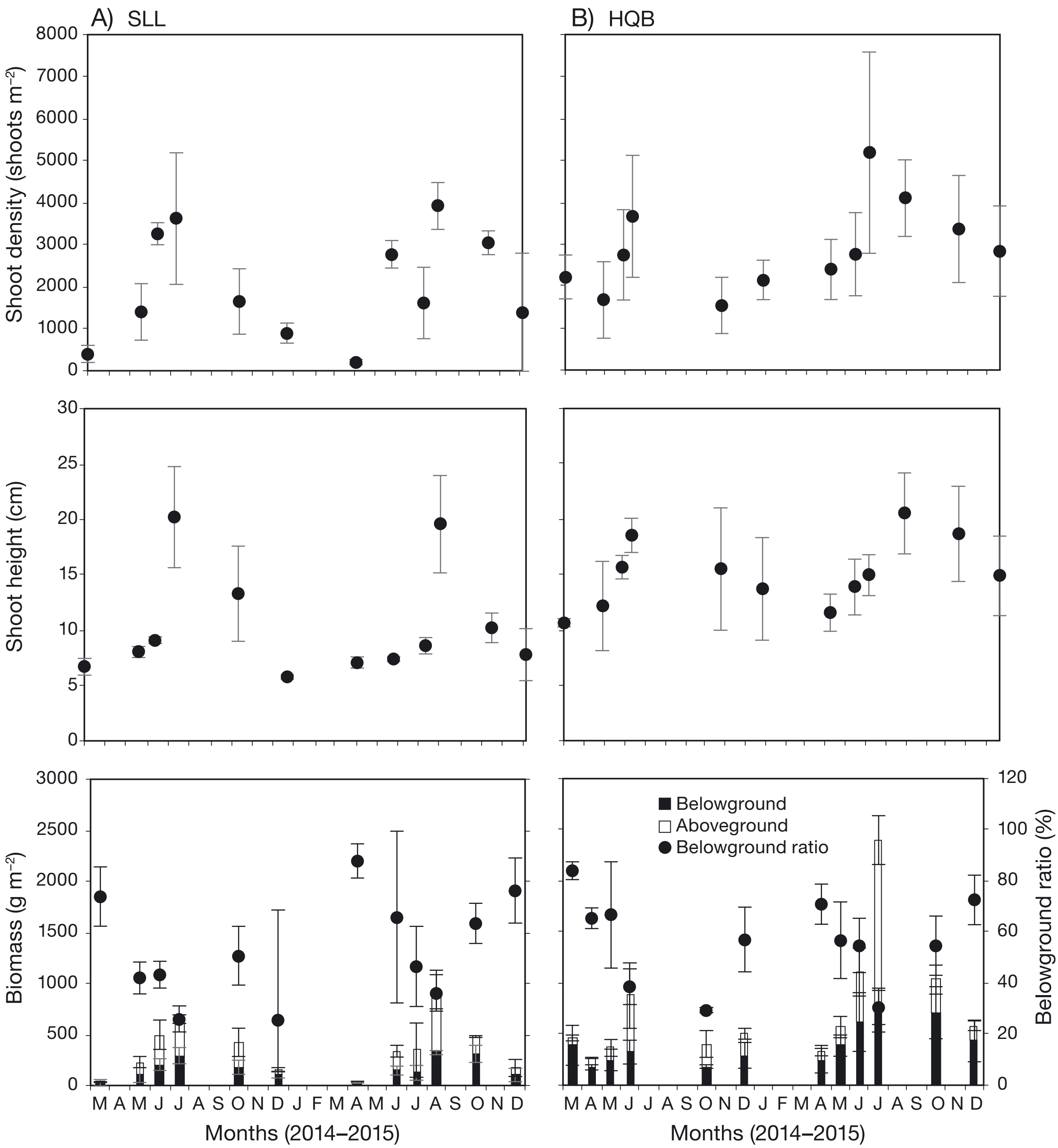

Fig. 2. Shoot density, shoot height, and biomass of *Zostera japonica* at (A) Swan Lake lagoon (SLL; n = 18) and (B) Huiquan Bay (HQB; n = 12) during 2014 and 2015 (means ± SD)

3.5. Seedling recruitment at SLL

Among the 10 permanent quadrats, 7 contained only seedlings and the other 3 were dominated by overwintering shoots (Fig. 6A). The first seedlings were observed by the end of March, when seedlings emerged in 9 out of 10 quadrats. Seedling density varied greatly among quadrats but averaged 1088 ± 1198 seedlings m^{-2} (Fig. 6A). Seedling density decreased by 38–100% in 7 of the quadrats over the next 3 wk, but increased by 20–50% in the other 2 quadrats; at the same time, internodes of seedlings were first observed. Seedlings began to produce new shoots by clonal growth in May, at which time seedlings only remained in 5 quadrats. Few new seedlings emerged after late May. The seedling

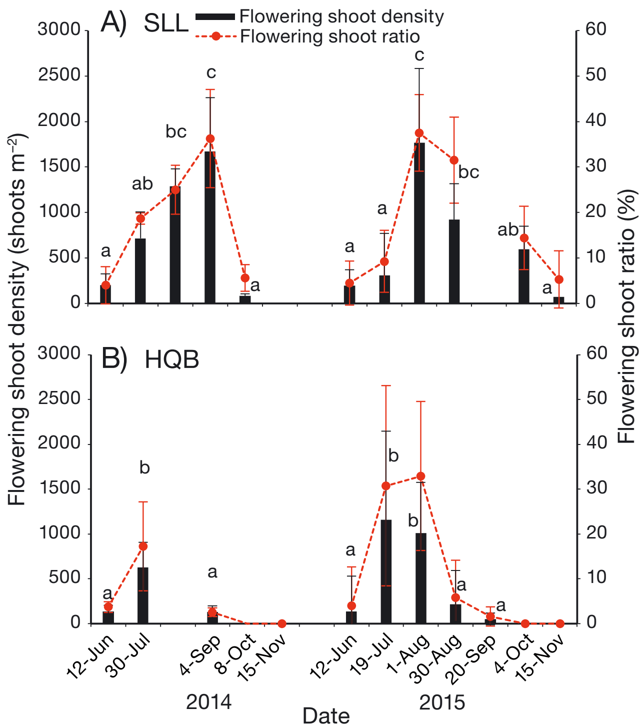

Fig. 3. Temporal changes in flowering shoot density and flowering shoot ratio of *Zostera japonica* at (A) Swan Lake lagoon (SLL; n = 18) and (B) Huiquan Bay (HQB; n = 12). Values are means ± SD. Means of density with different letters denoting significant differences between months within the same site ($p < 0.05$)

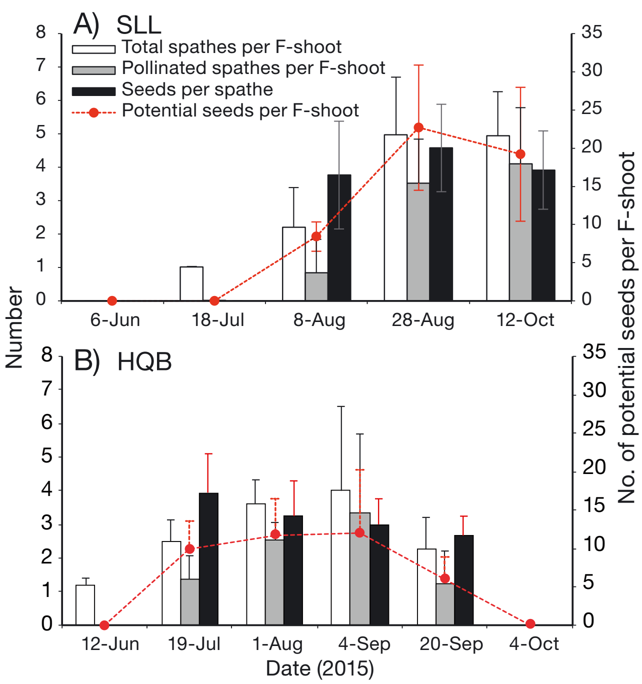

Fig. 4. Seed production per flowering shoot (F-shoot) of *Zostera japonica* at (A) Swan Lake lagoon (SLL) and (B) Huiquan Bay (HQB) (means + SD)

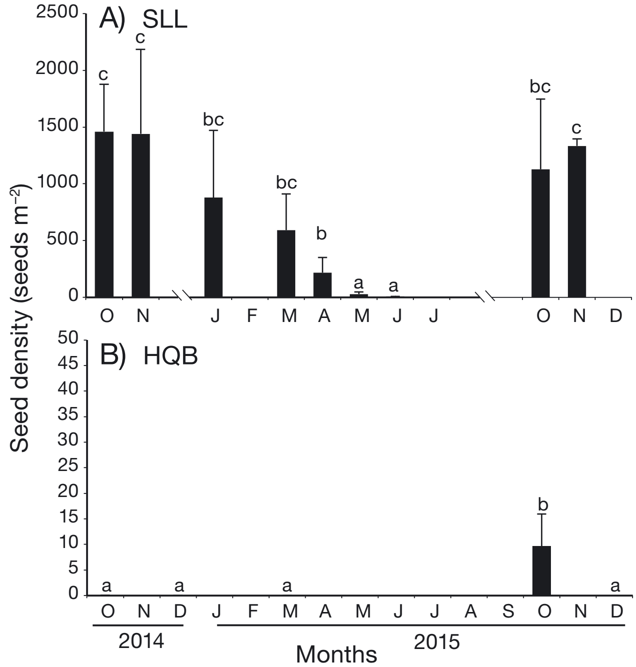

Fig. 5. Temporal changes in seed density in the sediment seed bank of *Zostera japonica* at (A) Swan Lake lagoon (SLL; n = 21–30) and (B) Huiquan Bay (HQB; n = 20–33) during October 2014 to November 2015. Values are means + SD; note the different *y*-axis scales of panels A and B. Means with different letters indicate significant differences among months within the same site ($p < 0.05$)

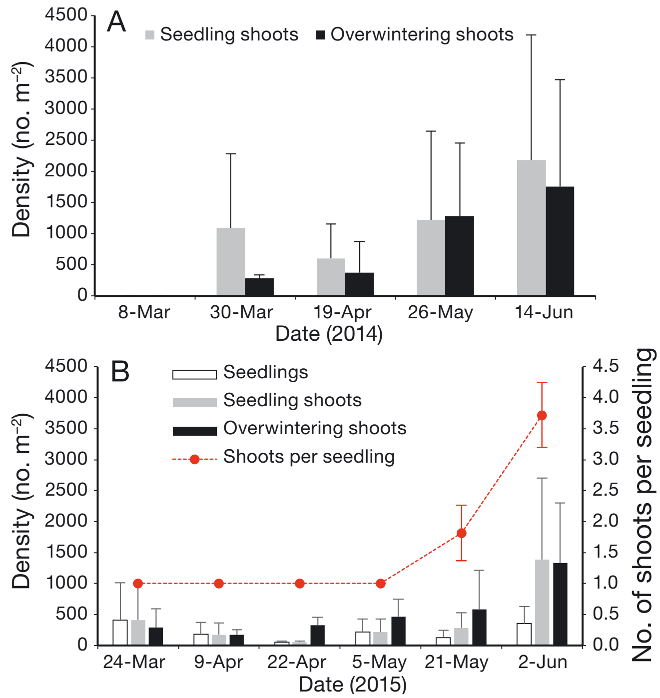

Fig. 6. Seedling recruitment and clonal growth of overwintering shoots based on (A) the permanent quadrats method in 2014 (n = 10) and (B) the random sampling method in 2015 (n = 18) at Swan Lake lagoon. Values are means + SD

shoot density was highest in the middle of June, with a mean of 2180 ± 2010 shoots m^{-2}. The density of overwintering shoots increased from 282 ± 58 to 1753 ± 1713 m^{-2} during spring (Fig. 6A).

In 2015, the dynamics of the seedlings identified by the random sampling method were similar to those in 2014 (Fig. 6B). The seedling density did not differ significantly among dates ($p > 0.54$), but the highest density (412 ± 601 seedlings m^{-2}) was found by the end of March. The final seedling density at the end of the germination period was 353 ± 279 m^{-2}, accounting for ca. 24 % of the maximum seed bank density in 2014. The seedlings began clonal growth in May, and the number of shoots per seedling increased from 2 ± 0 on 21 May to 4 ± 1 on 6 June. The final density of seedling shoots was 1389 ± 1314 m^{-2}. The minimum number of overwintering shoots (204 ± 62 shoots m^{-2}) was observed in early April. The overwintering shoots began clonal growth in late April, and the density increased to 1330 ± 968 m^{-2} in early June, which was the last time when seedlings and overwintering shoots could be distinguished by eye. The ratio of seedling shoots to total shoots did not vary significantly among dates, with a range of 21.84 ± 15.17 to 50.11 ± 49.39 %, and a final ratio of 41.16 ± 24.49 % at the end of the seed germination period.

3.6. Genetic and clonal diversity

The genotypic or clonal diversity at SLL reached the highest level (R = 1, Table 1), and all ramets (samples) collected in 2012 and 2015 belonged to a distinct genet. Genotypic diversity of HQB was much lower, with R = 0.66 in 2012 and R = 0.40 in 2015, and nearly 40 and 60 % of collected ramets originated from the same genets (Table 1). Table 2 shows the clonal structure in each patch at HQB. The number of ramets belonging to the same genet varied from 1 to 16. With the exception of new patches iv, v, and vi, all other patches had more than 1 genet. A total of 5 genets (G11, G12, G17, G22, and G29) were distributed in different patches. Old patches a and b shared 3 genets (G17, G22, and G29). New patch vii shared 1 genet (G11) with old patch c, and new patch ix shared 2 genets (G12 and G29) with old patch a. All genetic diversity indicators (i.e. H_o, H_e, PIC, and N_a; Table 1) were lower at HQB than at SLL, although these differences were not statistically significant ($p > 0.05$).

Table 1. Clonal and genetic diversity of *Zostera japonica* at Huiquan Bay (HQB) and Swan Lake lagoon (SLL) based on 7 microsatellite loci. N: number of ramets genotyped; G: genets identified from ramets; R: genotypic diversity [$R = (G - 1) / (N - 1)$]; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: polymorphic information content; N_a: mean number of alleles per locus; –: not calculated

Sites	Collection time	N	G	R	H_e	H_o	PIC	N_a
HQB	2012	34	23	0.66	0.5684	0.4720	0.5046	3.71
HQB	2015	84	32	0.40	0.5562	0.4170	0.5151	4.43
HQB-total		118	57	0.49	–	–	–	–
SLL	2012	40	40	1.00	0.5850	0.5476	0.5498	7.43
SLL	2015	24	24	1.00	0.6246	0.5536	0.5654	5.43
SLL-total		64	64	1.00	–	–	–	–

3.7. Comparison of the life history cycle between SLL and HQB

The life history cycles of the 2 populations differed mainly in the timing, duration, and quantity of the key events. Based on the field survey results, the life history cycle of the *Z. japonica* population at SLL can be quantitatively categorized into 4 main processes: seed germination and seedling establishment, recovery of overwintering shoots, flowering and seed production, and shoot decay (Fig. 7A). In addition, the seeds and residual shoots overwintered in the sediment and entered the next cycle (Fig. 7A). However, the process of seed germination could not be quantified at HQB due to the extremely low numbers of seedlings found (Fig. 7B).

4. DISCUSSION

This is the first comparative study of the life history strategies of a mixed-annual and a perennial meadow of *Zostera japonica* based on ecological surveys combined with microsatellite analysis. Different life history strategies of *Z. japonica* have been reported for both non-native and native locations, but previous studies paid little attention to seed production, seed bank, and especially seedling recruitment (Table 3). The 2 populations in this study exhibited quite distinct reproductive phenologies and quantitative characteristics in the key steps involved in the life cycle, especially in the stages of flowering and seed production, seed bank, and seedling recruitment.

The SLL and HQB populations differed in both flowering and seed production. The potential seed production

Table 2. Genets in patches of *Zostera japonica* at Huiquan Bay during 2015, showing the number of ramets genotyped (*N*), genets identified from ramets (*G*), and serial numbers of genets (G+number), where **bold** indicates that the genet occurred in more than 1 patch

Patch name	*N*	*G*	Genet composition
a (i)	25	16	G4+G8+G9+G10+**G12**+G16+**G17**+G19+G21+**G22**+G23+G24+G25+**G29**+G30+G31
b (ii)	7	5	**G17**+G18+**G22**+G26+**G29**
c (iii-1)	17	4	**G11**+G5+G20+G28
c (iii-2)	7	2	**G11**+G27
c (iii-3)	3	2	G1+G7
iv	3	1	G2
v	3	1	G15
vi	5	1	G32
vii	6	4	G3+**G11**+G33+G13
viii	5	2	G6+G34
ix	3	3	**G12**+G14+**G29**

per flowering shoot and per unit area were 2- to 3-fold higher at SLL than at HQB, although the density of flowering shoots did not differ between the 2 sites. Low reproductive output at HQB may be due to fewer spathes per flowering shoot and a lower seed-set compared to SLL. Based on the numbers of male and female flowers (4–5 vs. 4–7) in *Z. japonica* inflorescences (den Hartog 1970, Bigley 1981), the potential fruit set per spathe is 7 at most. The mode of seeds per spathe at SLL was 6, whereas it was only 2 at HQB. Pollen limitation is regarded as one common reason for a reduced seed-set in seagrasses (Van Tussenbroek et al. 2016), because failure of pollination is common due to rapid dilution of pollen and unpredictable hydrodynamic forces (Ackerman 2002). In addition, fragmented populations or isolated patches have a much lower seed-set, even if at similar flowering shoot density, than continuous populations (Reusch 2003, Van Tussenbroek et al. 2016). Therefore, we suggest that strong swells from the open sea (Chang et al. 1992) and the fragmented distribution of *Z. japonica* at HQB resulted in the lower seed-set.

Another important difference between SLL and HQB was in flowering phenology, including timing of initiation and duration of flowering and seed maturation. The HQB population began to flower 1–2 wk earlier, but the flowering duration was 2–3 mo shorter, than the SLL population. For terrestrial plants, temperature is considered to be the most important factor controlling timing of flowering, while photoperiod ranks second. This implies a strong effect of latitudinal position on phenology (Badeck et al. 2004, Forrest & Miller-Rushing 2010, Hut et al. 2013). Likewise, the start of flowering and seed maturation in *Z. marina* changes significantly with latitude and even more significantly with temperature (Blok et al. 2018), but it is less likely to be affected by photoperiod due to lack of a photoperiodic control related gene (Olsen et al. 2016).

Considering the similar latitudes and light availability of the 2 sites in the present study, higher temperature is likely the major reason for earlier flowering at HQB, which is also the case for *Z. marina* at HQB and SLL (S. C. Xu et al. 2018). However, populations of *Z. japonica* located at higher (~49° N) and lower latitudes (20~37° N) both showed an earlier and shorter flowering period (Table 3), although sometimes with annual variations, suggesting that other factors or interactions among factors may influence the flowering phenology of *Z. japonica*. In addition, the end, and thus, the duration, of flowering seemed unrelated to latitude and temperature (Blok et al. 2018), and the observed differences in flowering duration may represent different strategies of resource allocation.

The seed bank density at SLL was 2 to 3 orders of magnitude greater than at HQB (1460 ± 417 vs. 10 ± 6 seeds m^{-2}), and the 2 seed banks represented 3.6% and <0.1% of their seed production, respectively. This result indicates substantial losses of seeds from the 2 meadows and greater losses at HQB than that at SLL. The continuous strong swells from the open sea cause dynamic movements of sand at HQB (Chang et al. 1992), which likely cause the dispersal of *Z. japonica* seeds out of the meadow. The total area of the *Z. japonica* patches was limited at HQB, and thus, the absolute amount of seed production was probably negligible. In contrast, the seed bank density of *Z. marina* at HQB is relatively high (254 seeds m^{-2}) and comparable to that of *Z. marina* at SLL (268 seeds m^{-2}) (S. C. Xu et al. 2018). The different seed-retaining capacity between *Z. marina* and *Z. japonica* might be related to species-specific differences in seed size and mass, because heavy seeds tend to remain close to the parent plants while smaller seeds

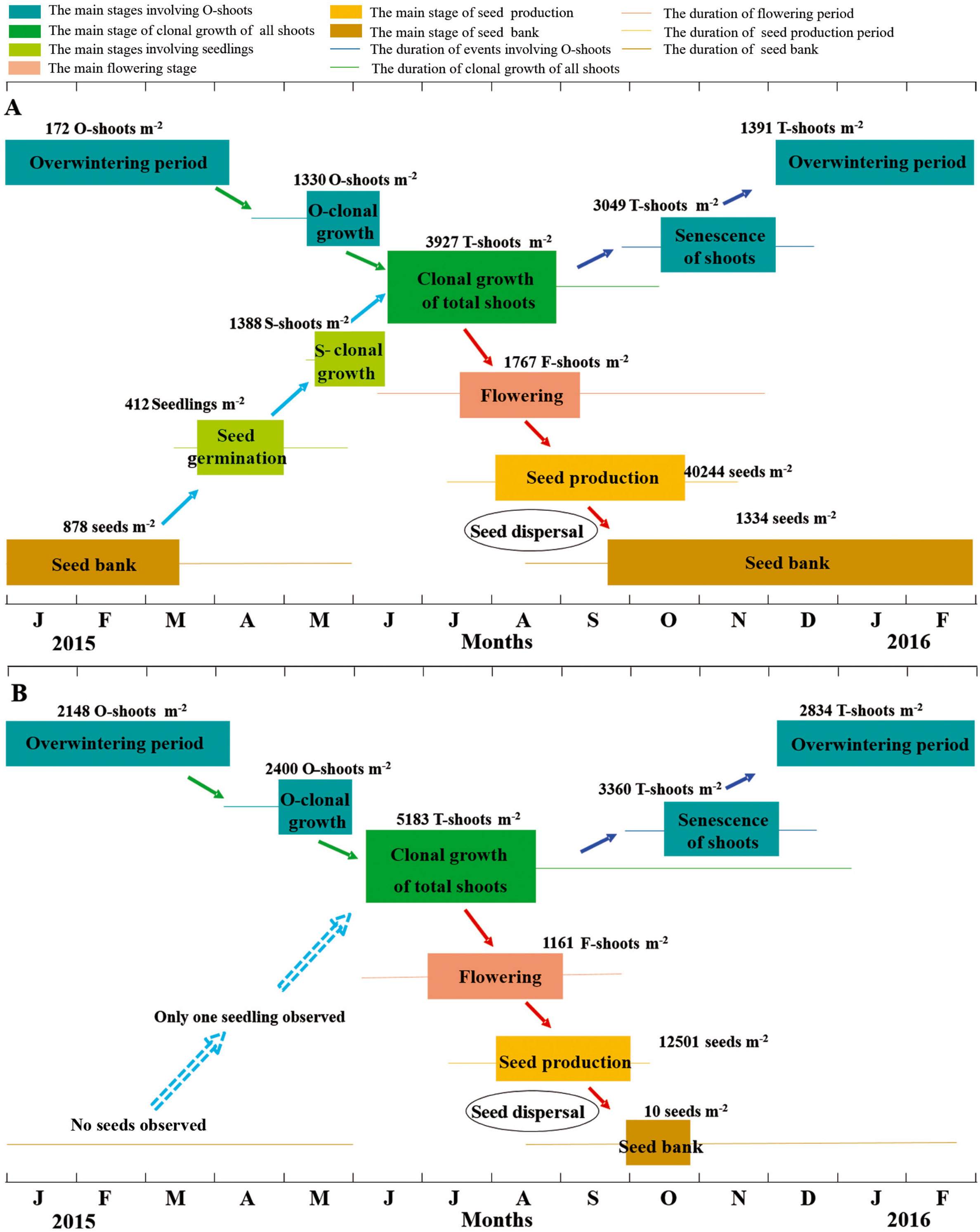

Fig. 7. Reproduction and recruitment cycle of *Zostera japonica* at (A) Swan Lake lagoon and (B) Huiquan Bay. The lengths of colored lines and rectangles correspond to time, i.e. the overall duration and main periods of different stages within the reproduction and recruitment cycle. O- and S-Clonal growth represent clonal growth by overwintering shoots and seedlings, respectively; O-, S-, T-, and F-shoot refer to overwintering shoots and shoots from clonal growth, seedling shoot, total shoot, and flowering shoot, respectively; values (means) are based on the results of the survey in 2015 and represent the peaks in each stage, except the value for overwintering shoots following the minimum after winter. Four processes are involved in this cycle: (1) seeds germinate from mid-March and seedlings begin clonal growth after mid-May; (2) O-shoots begin clonal growth from mid-April, which is later than germination; (3) in June, mixed S- and O-shoots begin rapid clonal growth, followed by flowering from June and seed output from July until November, when the seeds are dispersed into the sediment (seed bank); (4) shoots that do not flower decline over time but some overwinter and enter the next cycle

Table 3. Review of studies related to the life history events of *Zostera japonica* throughout its range. O-shoot: shoot produced from overwintering rhizomes; F-shoot: flowering shoot; –: no data

Location	Coordinates	Growth form	O–shoot density (shoots m^{-2})	Flowering period	F–shoot density / ratio (shoots m^{-2}) / (% where indicated)
Boundary Bay, Canada	49°02′N, 123°08′W	Mixed–annual	–	May–Sep (1978)	215 (Aug 1978)
			Negligible	May–Sep (1979)	169 ± 25 (Aug 1979)
Fraser River Delta, Canada	49°02′N, 123°06′W	Mixed–annual	Few	Jun–Oct (1979); Jul–Dec (1980)	~1600 (Aug 1979); ~1200 (Aug 1980)
Willapa Bay, USA	46°35′N, 124°02′W	Annual and perennial	<500 ~ <3000 (Feb 2004)	Jun–Oct (2004)	– /13–34 % (mean = 30 %; Jun~Sep 2004)
Yaquina Bay, USA	44°38′N, 124°01′W	Perennial	1500 (Feb 2002)	~Dec (2001); Jul–Oct (2002)	461 ± 206 / 10.2 ± 11.4 % (Sep/Oct 2001); 170 ± 408 / 2.5 ± 4.1 % (Jul/Oct 2002)
			–	Jun–Nov (2011–2012)	–
Yellow River estuary, China	37°48′N, 119°10′E	Mixed–annual	30 ± 30 (Mar 2016) ~ 162 ± 206 (Mar 2017)	Jun–Oct (2015, 2016)	904 ± 59~2700 ± 369 (Aug 2015); 794 ± 279~2919 ± 727 (Aug 2016)
Swan Lake lagoon, China	37°21′N, 122°34′E	Mixed–annual	403 ± 205 (Mar 2014); 204 ± 62 (Apr 2015)	Jun–Oct (2014); Jun–Dec (2015)	1674 ± 591 / 36.3 ± 10.8 % (Sep 2014); 1767 ± 819 / 37.5 ± 8.4 % (Aug 2015)
Seungbong Islanda, Korea	37°09′N, 126°17′E	Perennial	2500~5000	Jul–Oct/Nov (2002)	~1300; 5~20 % (Jul~Aug 2002)
Huiquan Bay, China	36°05′N, 120°34′E	Perennial	2220 ± 522 (Mar 2014); 2400 ± 716 (Apr 2015)	Jun–Sep (2014, 2015)	625 ± 281 / 17.3 ± 9.9 % (Jul 2014); 1161 ± 986 / 32.9 ± 16.7 % (Jul/Aug 2015)
Koje Bay, Korea	34°48′N, 128°35′E	Perennial	4024 (Jan 2003); 1321 (Feb 2004); 3387 (Jan 2005)	May–Jun (2003); Mar–Aug (2005)	2293 / 21 % (May 2003); 5867 / 63 % (Mar 2005)
			1681 ± 123 ~ 2547 ± 293 (Jan–Feb 2016)	May–Jul/Aug; May–Aug/Sep; May–Oct (2015–2016)	201 ± 31~1248 ± 177 / 6.6 ± 4.1 ~27.2 ± 4.0 % (Jun–Jul 2016)
Dadae Bay, Korea	34°43′N, 128°37′E	Perennial	>6000	May–Aug	900 / 10 % (late Jul 2001)
Lantau Island, Hongkong, China	22°17′N, 113°55′E	Perennial	–	Mar–May	–
Ha Long Bay, Vietnam	20°51′N, 106°59′E	Perennial	~1800 (Feb 2001)	Apr (2001)	400 / 9 % (Apr 2001)

Table 3 (continued)

Seed production (seeds m^{-2})	Seed bank (seeds m^{-2})	Germination period	Seedling density (seedlings m^{-2})	Recruitment from seeds	Reference
–	Many	–	–	Mainly	Harrison (1979)
–	–	Mar/Apr~?	–	Most	Harrison (1982a)
–	–	~Jun (1979); Mar–Jul (1980)	~120 (Apr 1979 & May 1980)	~>90 %	Harrison (1982b)
–	–	Mar–Jun (2004)	–	12–57 % (mean = 30 %; Mar–Apr 2004)	Ruesink et al. (2010)
–	–	–	–	–	Kaldy (2006)
–	1988 ± 1074 (Sep 2012, site HF)	Sep–Apr	785 ± 1202 (Jan 2011, site DB)	–	Henderson & Hacker (2015)
13137 ~ 30784 (2015)	1773 ± 802 (Dec 2015); 2382 ± 1606 (Oct 2016)	Apr–May (2016, 2017)	343 ± 395 (May 2016) – 3084 ± 716 (May 2017)	35.46 ± 34.36 % (2016) – 96.51 ± 5.51 % (2017)	Zhang et al. (2019)
40244 ± 18666 (2015)	1461 ± 417 (2014); 1068 ± 1046 (2015)	Mar–Jun (2014, 2015)	411 ± 601 / 184 ± 192 (Mar/Jun 2015)	41.16 ± 24.48 % (Jun 2015)	This study
–	–	–	–	–	Lee et al. (2005)
12501 ± 5748 (2015)	10 ± 6 (Oct 2015)	Unknown	Unknown	Only 1 seedling observed	This study
–	–	–	–	–	Park et al. (2011)
2177 ~9737 (2015~2016)	76 ± 31~54 ± 166 (2015–2016)	Unknown	Unknown	Not observed	Suonan et al. (2018)
–	–	–	–	–	Lee et al. (2006)
–	–	–	–	–	Lee (1997)
–	–	–	–	–	Huong et al. (2003)

are more likely to disperse further (Delefosse et al. 2016). Seeds moving out of meadows have been directly observed at SLL, where *Z. japonica* seeds were found in the *Z. marina* zone as far as 40 m from to nearest margin of a *Z. japonica* zone (X. M. Zhang et al. unpubl. data). However, natural mortality and predation may also account for considerable losses of seeds from the seed bank (Harrison 1993, Fishman & Orth 1996, Sumoski & Orth 2012).

The most striking difference in the life history cycle of *Z. japonica* between HQB and SLL was in the process of seedling recruitment. Less than 24 % of the initial seed bank at SLL transformed into established seedlings and contributed to population recruitment. At SLL, the general process and key timing of seed germination and seedling establishment, and the final contribution of seeds to population recruitment were clearly defined and quantified based on ecological observations. In contrast, although flowering and ripening of seeds were common at HQB, only 1 seedling was observed in June, which indicated that spring germination was similar to that at SLL, but the seedling recruitment process still remains obscure. Similarly, although flowering and seed bank size were noticeable, no seedlings were found in the perennial *Z. japonica* meadows in Koje Bay, Korea (Suonan et al. 2017). As no seeds remained in the sediment from March to May, it was posited that the seeds had germinated, but that the seedlings were too small or too easily broken to be found during sampling (Suonan et al. 2017). This might also explain the lack of seedlings at HQB, but a more obvious reason could be the extremely small seed bank at HQB, where the seeds disappeared rapidly after release. In addition, interference from the relatively high shoot density of *Z. japonica* and *Z. marina* throughout the year could have constrained the germination rate and early seedling establishment (Lee et al. 2007).

The seedling contribution to population recruitment at SLL corresponded to 41 ± 24 %, which indicates a contribution comparable to that of clonal growth in overwintering shoots. Based on a limited number of studies, the significant contribution of seedlings for population recruitment seems common in mixed-annual populations (Harrison 1979, 1982b, Zhang et al. 2019), where the contribution of seeds can increase to >90 % at higher latitudes (49° N) (Harrison 1979, 1982a,b) or in the mid-latitudes when encountering an extremely cold weather (Zhang et al. 2019). Substantial recruitment via seeds was also observed in a perennial population of *Z. japonica* in Yaquina Bay, USA, where seeds germinated from September to April of the next year, peaking in November to January (Henderson & Hacker 2015). It was suggested that sexual reproduction in Yaquina Bay was a potential escape response to increasing sediment disturbance (Henderson & Hacker 2015).

Genetic diversity and structure in populations of clonal plants are shaped by interactions between sexual reproduction and clonal growth (Widén et al. 1994, Waycott et al. 2006). As only 1 seedling was observed at HQB, it seemed that seeds contributed little, and thus, clonal growth was the prime mechanism by which populations were maintained. However, the genetic diversity and clonal structure of the meadow provided new clues to understanding the contribution of the 2 types of reproduction to population recruitment. Clonal diversity (R) was relatively high ($R = 1$) at SLL, meaning that no ramets were collected repeatedly and indicating high genet turnover and frequent seedling recruitment (Zipperle et al. 2009b). In contrast, clonal diversity at HQB was relatively lower ($R = 0.66$ and 0.40 at a sampling interval of >2 m and ≤1.0 m, respectively), suggesting that 34 and 60 % of ramets were repeatedly sampled. This result verifies the important role of clonal growth in maintaining populations at this location. Moreover, we found that several genets were distributed simultaneously in different patches and even among the old and new patches. On one hand, this result suggested that asexual reproduction contributed to the formation of new patches at HQB likely via vegetative propagules, which highlights other important means of dispersal and colonization in aquatic plants besides seed dispersal (Berkovi et al. 2018). On the other hand, it indicated the role of asexual reproduction in maintaining genetic diversity through expansion of clones among years and increasing chances of gene flow among patches. However, genetic diversity in terms of H_o and H_e at HQB was relatively high, although slightly lower compared with that at SLL. This might be because repeated seedling recruitment still could increase the population genetic diversity even though the number of recruited seedlings in clonal plants in the field was often very low (Soane & Watkinson 1979). Our findings illustrate the role of sexual reproduction in sustaining genetic diversity in perennial populations, although at an extremely low frequency.

5. CONCLUSIONS

Results of this study provide insight into the different roles of sexual and asexual reproduction for populations of different growth forms (annual vs. perennial). The contribution of seedling recruitment to annual populations is relatively high, but for most perennial populations, seedlings are difficult to observe, and thus, the contribution of sexual recruitment is often neglected. Molecular markers such as microsatellites and single nucleotide polymorphisms provide alternative ways to study the role of sexual reproduction in perennial populations. There is increasing interest in the restoration of seagrass meadows in areas where they have been lost. This study showed the role of sexual and asexual reproduction in ecological maintenance and evolutionary connectivity of seagrass populations, and the results emphasize the need to understand the local recruitment strategies before starting restoration and management projects.

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