

Genetic recolonization of mangrove: genetic diversity still increasing in the Mekong Delta 30 years after Agent Orange

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ABSTRACT: The widespread use of Agent Orange (a mixture of phenoxy herbicides) over Southern Vietnam by US Forces led to the decimation of mangrove forests in the Mekong Delta. Mangrove trees *Avicennia alba* were sampled across the Mekong Delta; their age was assessed using models based on internode growth and samples were genotyped for 6 microsatellite loci. The evolution of genetic diversity over time elapsed since local extinction was reconstructed and compared with the genetic diversity of an unaffected population from Thailand. The results show that genetic diversity of the *A. alba* population is still increasing in the Mekong Delta 3 decades after the end of the Vietnam War, but is reaching an asymptotic level that is comparable to the adjacent non-affected population of Thailand. This might be a sign of genetic recovery, but may also reveal a limitation, either of genetic enrichment due to current predominance of auto-recruitment or of demographic increase due to intraspecific competition in this pioneer species. In any case, these results, although encouraging, demonstrate that genetic recovery after complete or almost complete population depletion continues over a longer time-scale than apparent demographic recovery.

KEY WORDS: Recolonization genetics · Genetic recovery · Demographic recovery · Local extinction · Mangrove · Deforestation · Agent Orange

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INTRODUCTION

Mangrove ecosystems are one of the world's most valuable (Costanza et al. 1997) and most threatened (Valiela et al. 2001) ecosystems. Mangrove ecosystems are declining globally at rates of about 2.1% yr⁻¹ (Valiela et al. 2001), the major causes of this loss being replacement of mangrove areas by aquaculture ponds, logging for wood and charcoal, and reclamation (Costanza et al. 1997, Mumby et al. 2004). Mangrove disappearance leads to increased vulnerability of coastal

areas to flooding and storms and loss of harvestable resources and biodiversity. Mangrove regression has been particularly acute in Asia, where 36% of the original area covered by mangroves has been lost (Fortes 1988, Valiela et al. 2001). Realization of the loss of valuable ecosystem services associated with mangrove decline has led to the implementation of large-scale afforestation programs in SE Asia (Hong & San 1993, Hong 1996).

The largest single event of mangrove loss and subsequent reforestation was related to the widespread use

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by US Forces of a highly toxic defoliant named Agent Orange (Stellman et al. 2003) over Southern Vietnam, which led to the decimation of mangrove forests in the Mekong Delta. A major unprecedented reforestation program was led, following the end of the US–Vietnam war (1974), by the Vietnamese government to recover the lost mangrove area (Hong & San 1993, Hong 1996). This effort, however, was based on the use of a single genus, *Rhizophora*, for which nurseries were available and which local communities (including scientists and local farmers) believed to be the most valuable component of mangrove forests. As a result of these efforts, *Rhizophora* stands have partially recovered (Hong 1996). In contrast, the recovery of other mangrove species in the community, such as the hermaphroditic *Avicennia alba*, was dependent on propagule dispersal from external sources, but tree cover and density were recovered about 2 decades later (Hong 1996). However, because potential sources of propagules in the Mekong Delta were few and distant, due to the thorough herbicide devastation of the vast mangrove forest area, it is likely that most colonizing propagules were delivered from a few sources. As a consequence of this extinction–recolonization process, the recovery of the plant population may have involved initial decimation of genetic diversity relative to the original stands, a genetic bottleneck effect, eventually followed by recovery at an unknown rate. Whereas the rates and patterns of recovery of plant communities have been extensively studied, including that of the Mekong Delta (Hong 1996), there is a paucity of information on the rates of recovery of genetic diversity. Although some animal populations have recovered rapidly after drastic bottlenecks or local extinction (Barber et al. 2002, Charbonnel et al. 2002, Colson & Hughes 2004), reduced genetic diversity may persist much longer in some tree populations exhibiting low dispersal and high variance in reproductive success among parents in the source population (Sezen et al. 2005).

In the present study we reconstructed the rate of genetic recovery of the hermaphroditic mangrove *Avicennia alba* population in the Mekong Delta 3 decades after the end of the Vietnam War. The case of *A. alba* is particularly relevant because: (1) it is an important member of the Mekong Delta mangrove community; (2) recovery took place naturally (not planted); and (3) *Avicennia* species are characterized as pioneer species, the first to colonize empty sea fronts, and thereby are particularly relevant for a recolonization study conducted over the first decades post-disturbance, since these species may provide a conservative estimate of the time needed for recovery, expected to take even longer for later successional species. Genetic recovery was reconstructed based on a combined assessment of

plant age, used to estimate colonization time, and genetic diversity as estimated by the allelic richness at microsatellite loci (Teixeira et al. 2003). We considered 2 possible scenarios for the evolution of genetic diversity during the recolonization process. If, despite the complete devastation of the plant community portrayed by records immediately following the war (Hong & San 1993, Hong 1996), recolonization is mostly driven by the reproduction of local individuals that survived the event, genetic diversity would be related to the bottleneck size and we do not expect allelic richness to increase significantly with time over a scale of decades, for which mutation effects are negligible. Alternatively, recolonization driven by the repeated settlement of seeds imported from distant sources would lead to a progressive increase in allelic richness over time.

MATERIALS AND METHODS

Study site and plant age determination. In April 1998 we sampled 3 *Avicennia alba* stands originated by natural recovery in each of 2 areas of approximately 1 ha — Forest Park (10° 30.21' N, 106° 52.34' E) and Dan Xay (10° 24.02' N, 106° 52.60' E) — both located in the Can Gio National Park in the Mekong Delta, Vietnam, a region that was totally deforested by Agent Orange. Agent Orange and other herbicides were spread in Vietnam between 1962 and 1971, with the bulk of the chemicals applied between 1966 and 1969 (Stellman et al. 2003). Leaf samples were collected from 232 trees ranging from saplings to the largest trees (25 m in height, 1.92 m in girth) for genetic analyses.

The age of all sampled plants was determined from the number of internodes the plant produced throughout its life span (Duarte et al. 1999), or from linear regression equations developed in the present study between plant age and height (Coulter et al. 2001). The internodes present along mangrove stems are characterized by a cyclical pattern of internodal length, with the shortest internodes produced in winter and the longest in summer, such that the number of internodes produced in a year is relatively constant and independent of plant age. We therefore counted the number of internodes, which allows the estimation of plant age, and we measured the height of all plants sampled. The number of internodes of the trees large and old enough for secondary bark growth to mask the internodes, or for the highest internodes to be beyond our reach, could not be counted. Therefore, the age of these trees was estimated from linear regression between tree age (yr) and height (H; cm), developed by pooling data for all trees for which age was determined from the number of internodes produced

(Figs. 1 & 2). The fitted regression equation was $\text{Age} = 0.92 + 0.008H$ ($R^2 = 0.78$), and the standard error of the estimates was ± 0.65 yr. In order to establish the average number of internodes produced annually along the stems of the sampled *Avicennia alba* stands, we measured the sequence of internodal length along the main stems of 16 plants. We calculated the number of internodes in each cycle (i.e. year) and then estimated the average (\pm SE) number of internodes produced per year to be 11.38 ± 0.33 internodes, somewhat lower than derived for more southern populations in Thailand (Duarte et al. 1999). We therefore estimated the plant age as the ratio between the number of internodes present along their main stem and the number of internodes produced per year, yielding an estimated uncertainty of $\pm 5\%$. In order to estimate the age of plants for which the number of internodes could not be counted, we used a linear regression equation, fitted using reduced major axis type II regression (Draper & Smith 1966), between estimated plant age and height ($\text{Age} = -0.61 + 0.014H$, $R^2 = 0.74$, $p < 0.001$).

In addition, we thoroughly searched the potential mangrove habitat upstream of the impacted area to sample the oldest, scattered *Avicennia alba* trees ($N = 18$) in order to characterize the genetic diversity of the population prior to or immediately following the disturbance. Age determinations indicated that only 4 of the trees located were present before the disturbance and 14 immediately after. Finally, trees ($N = 47$) were sampled in an unaffected forest from southern Thailand in

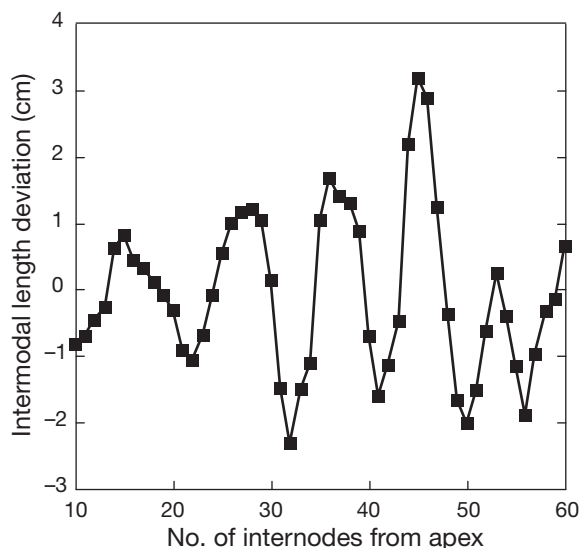


Fig. 1. *Avicennia alba*. A sample sequence of deviations from the mean internodal length for internodes from the tip to the base of a tree sampled at Forest Park in Can Gio, Mekong Delta, Vietnam. Data represents the residuals obtained after subtracting the raw estimates of internodal length from the running average of 15 internodes, to remove long-term, interannual trends

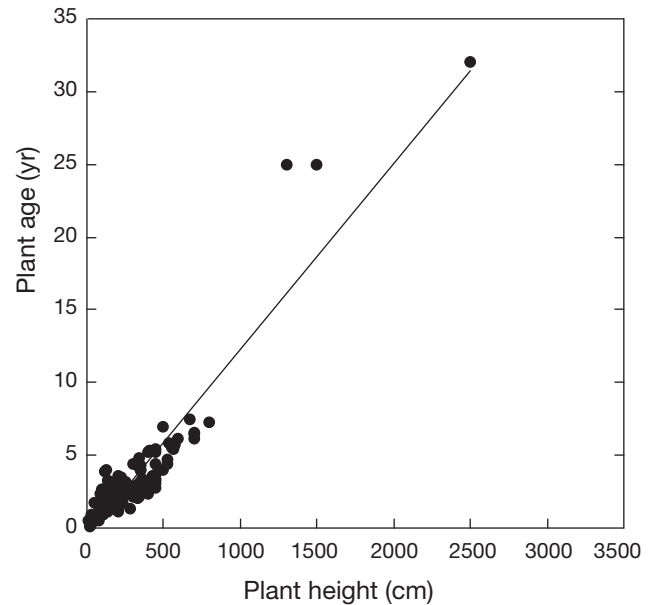


Fig. 2. *Avicennia alba*. Relationship between the estimated age of trees sampled across the Mekong Delta, Vietnam, and their height. The solid line shows the fitted linear regression

order to compare the levels of diversity and departures from linkage and Hardy-Weinberg equilibrium (HWE) in the current populations of the Mekong Delta with those from an unaffected area.

Genetic methods. Genomic DNA was extracted from leaf tissue using the CTAB method (Doyle & Doyle 1987). Six polymorphic microsatellites (Teixeira et al. 2003) were amplified by PCR as described by Teixeira et al. (2003). PCR products were separated in 6% denaturing polyacrylamide gels and visualized by autoradiography.

The recovery rate of genetic variability was characterized as the increase in the average number of alleles in the *Avicennia alba* population at 5 yr intervals since the disturbance in trees recruited before 1978, 1983, 1988, 1993 and 1998. This was done both for the cumulated age class, in order to mimic the evolution of allelic richness (A) in the population over time, and on each age class separately, to understand whether the allelic richness was evolving due to a cumulative effect of increasing recruitment events or an increase in the genetic diversity of recruits over time. In order to account for the decreasing sample size with time elapsed before the study was conducted, allelic richness was computed by resampling (1000 iterations) from each combined age class to maintain a homogeneous sample size ($N = 18$, the smallest observed sample size corresponding to the oldest age classes for the cumulated age class; $N = 17$, the smallest effective sample size for the 15–20 age class), using GenClone software (Arnaud-Haond & Belkhir 2007). Multilocus

genotypes were tested for deviations from HWE using F_{IS} estimates, and for linkage disequilibrium with the 2-locus correlation coefficient R^2 (Weir 1979) estimated as described by Black & Krafur (1985), using the Genetix 4.0 package (Belkhir et al. 2001). The estimates were performed for each site as well as on the overall sample for each 5 yr interval since the disturbance, and significance was tested by a 1000 permutation test.

RESULTS

The age of the *Avicennia alba* plants genotyped ranged from about 1 mo to 40 yr. The oldest plant sampled (recruitment estimated in 1959) recruited before the spread of Agent Orange was initiated, and 2 additional plants (age range 30 to 32; i.e. recruited between 1967 and 1969) dated from before complete devastation by the Agent Orange application. These 3 plants were located upstream on the watershed in areas presently occupied by crops. The remaining plants were all found within the Can Gio National Park, in areas devastated between 1961 and 1971 by Agent Orange, and were therefore representative of the product of natural recolonization processes. The 232 trees sampled in 1998 were split into 5 age classes. The oldest class (<1978), including 18 trees between 20 and 40 yr old, was composed of the 3 remnant trees and 15 more that recruited during the first 5 yr after the defoliation. The rest of the sample was split into age classes of 5 yr (1978–1983, 1983–1988, 1988–1993 and 1993–1998). Those classes were analyzed both individually and cumulatively as samples of the population along the recolonization process, at each 5 yr step (making classes of trees germinated before 1978, 1983, 1988, 1993 and 1998).

Global allelic richness standardized for sample size showed a significant increase since the end of the war in 1973 ($R^2 = 0.96$, $p = 0.002$; Fig. 3), increasing by 14 % over 25 yr. Yet the rate of increase in allelic richness declined from a maximum of $1\% \text{ yr}^{-1}$ in the mid-1980s to a marginal increase of $0.14\% \text{ yr}^{-1}$ 3 decades after the disturbance. The standardized allelic richness reached 4.75 ± 0.09 alleles loci^{-1} across the Vietnam sampling sites, whereas the standardized allelic richness estimated in the sample from the unaffected Thai population was about 5.4 ± 0.06 alleles loci^{-1} . Most alleles present in the Vietnam sample, and all the most common ones, were shared with the Thai sample.

When analyzed in each age class separately (i.e. non-cumulative), the allelic richness did not show a

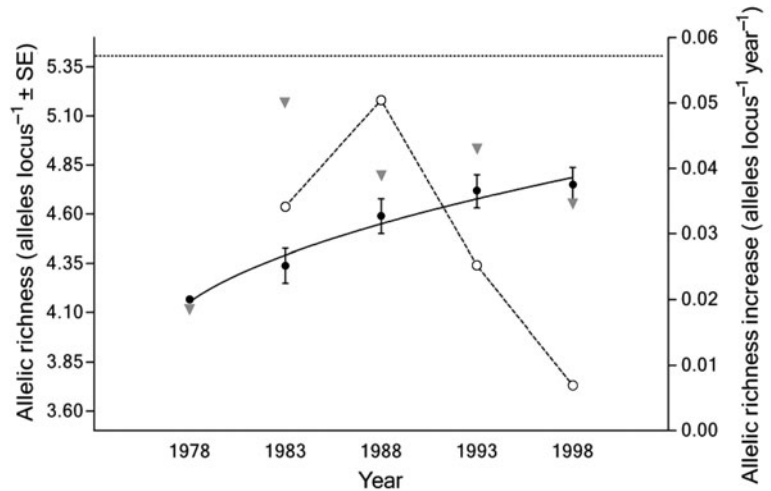


Fig. 3. *Avicennia alba*. Temporal increase (filled symbols) and the rate of increase (open symbols) of genetic diversity (as average allelic richness \pm SE) at 6 microsatellite loci) since the end of the Vietnam War, in trees in Can Gio, Southern Mekong, Vietnam. Years on the x-axis indicate the upper boundary of each age class. Black circles illustrate cumulated allelic richness and grey triangles the allelic richness in each age class. Estimates of standardized allelic richness (\hat{A}) are standardized for the minimum sample size ($N = 18$ for the cumulated age classes, $N = 17$ for the age classes taken separately). The solid line shows the fitted regression equation: alleles $\text{loci}^{-1} = 3.8 (\pm 0.07) + 0.20 (\pm 0.02) \times t^{0.5}$ (where t is the years elapsed since 1975; $R^2 = 0.96$, $F = 99$, $p = 0.0024$). The horizontal dotted line indicates the average allelic richness (based on subsampling of $N = 18$) in the Thai sample

linear progression in time, but rather an irregular pattern, with the lowest allelic richness found in the oldest age class and the highest in the samples from the second oldest age class (Fig. 3).

No heterozygote deficiency (Table 1) was observed in the oldest (germinated <1978) class of trees ($F_{IS} = -0.02$, $p > 0.05$) sampled over the Mekong Delta, nor in the sample from Thailand ($F_{IS} = 0.06$, $p > 0.05$). Heterozygote deficiency appeared in the population during recolonization, as attested by the significant values observed in cumulated age classes ($F_{IS} = 0.04$ to 0.22 ; Table 1). These departures from HWE appeared on an increasing number of loci reaching 5 loci out of 6 with significant departure in the entire set of samples over the entire recovery period studied (all cumulated age classes). In the same way, no significant linkage disequilibrium (LD) was detected in the oldest class of trees, or in samples from Thailand, whereas some significant LD values were observed in the cumulated age classes in Vietnam. In the sample from the population as present in 1983, significant ($p < 0.05$) LD values appeared among 2 pairs of loci (Am13-Am26 and Am26-Am67), as in the population present in 1988 (Am22-Am28 and Am13-Am26). One pair of loci showed significant LD values in the sample of trees present in 1993 (Am22-Am28), as did 3 pairs of loci in the sample of the 1998 population of the Mekong Delta (Am22-Am28, Am23-Am26 and Am23-Am67).

Table 1. Departure from Hardy Weinberg equilibrium in each age class of each of the 2 *Avicennia alba* stands sampled (Forest Point and Dan Xay) and after pooling all the samples collected across the Mekong Delta (Overall). F_{IS} estimates, as well as the number of trees they were computed from (N), are given for the populations at each 5 yr time step from 1978 to 1998, and for the Thai population sampled in 2002. **: $p < 0.01$; *: $p < 0.05$; ^{NS}: $p > 0.05$; n: number of loci with significant F_{IS} values

	Year	Forest Point			Dan Xay			Overall		
		N	F_{IS}	n	N	F_{IS}	n	N	F_{IS}	n
Vietnam	1978	–	–	–	–	–	–	18	0.02 ^{NS}	0
	1983	7	0.23*	3	6	0.04 ^{NS}	1	35	0.11**	2
	1988	25	0.12*	1	18	0.20**	1	65	0.14**	4
	1993	44	0.10**	2	42	0.12**	2	108	0.12**	4
	1998	103	0.08**	2	107	0.17**	4	232	0.14**	5
Thailand	2002	–	–	–	–	–	–	47	0.06 ^{NS}	1

DISCUSSION

The present study shows that genetic diversity of *Avicennia alba* in the Mekong Delta has been progressively increasing since the destruction by Agent Orange, although its slow rate of increase in the mid-1990s suggests that genetic diversity was reaching an asymptotic level 3 decades following the end of the catastrophic disturbance experienced (Fig. 3). However, it is not possible to ascertain whether genetic recovery is complete due to the absence of records prior to disturbance. The allelic richness present in Vietnam at the beginning of recolonization was about 75 % of the standardized allelic richness estimated in Thailand, and is now reaching about 80 %. Although based on a limited sample size for the oldest age classes, a pitfall that is a direct consequence of the subject of the present study—the almost complete decimation of the mangrove forest—this comparison only suggests that the present day Vietnamese populations of *A. alba* have reached a level of allelic richness comparable to that of an unaffected population. However, the comparison cannot be extrapolated further as there is no indication that the effective population size of the Thai population would be comparable to that of the Vietnamese population before disturbance.

Departures from HWE also support the occurrence, following disturbance, of a still ongoing recolonization process from distinct external sources. Significant F_{IS} values can have a variety of origins, and are influenced by both technical and biological factors. Technical factors such as null alleles or preferential amplification usually result in locus-specific patterns of departure from HWE (Zouros & Foltz 1984, Hare et al. 1996). The occurrence of selective processes on the markers genotyped or on some tightly linked gene would also result in locus-specific patterns (Gaffney 1994), which is not the case here. Other biological explanations, including the Wahlund effect (Wahlund 1928) or inbreeding,

affect the genome as a whole and are therefore expected to result in rather homogeneous departures from HWE over loci (Zouros & Foltz 1984). In the present study, departures from HWE did not occur in the undisturbed population of Thailand, nor were they observed in the eldest sample from Vietnam. These results, together with the increase in F_{IS} over time, reaching significant and positive F_{IS} in 5 out of 6 loci over all Vietnamese samples (Table 1), strongly support the hypothesis of a biological origin of those departures from HWE, the effect of which increases with time during the process of colonization.

Moreover, the increase in LD in the most recent age class of Vietnamese trees is also in agreement with the occurrence of a spatial and temporal Wahlund effect as well as the occurrence of inbreeding. Small sample size may limit the statistical power to reveal significant departure from HWE or LD in the eldest group of trees sampled in Vietnam. However, the lack of significance and also the lack of a trend (with $F_{IS} = -0.02$ compared to values reaching 0.10 to 0.15 in recent age classes, and same qualitative result observed for LD) suggest that this oldest sample of trees is derived from a single panmictic population spanning the Mekong Delta prior to defoliation. The same result obtained from the Thai population sample points to panmixia as a likely state in the natural and undisturbed population of this species. The subsequent departures from HWE and linkage equilibria support the hypothesis of recolonization of the Mekong Delta from several genetically distinct sources, or the occurrence of spatial structure (i.e. temporal or spatial Wahlund effect), as well as possible local inbreeding. The occurrence of a spatial and temporal Wahlund effect is one of the classical hypotheses put forward to explain the genetic patchiness (Johnson & Black 1982, 1984) in the marine environment, a phenomenon increasingly reported both for invertebrates (Jolly et al. 2003, Juinio-Menez et al. 2003, Casu et al. 2005, Virgilio & Abbiati 2006, Virgilio et al. 2006, Andrade & Solferini 2007, Arnaud-Haond et al. 2008) and fish (Doherty et al. 1995, Exadactylos et al. 1998, Planes et al. 2002, McPherson et al. 2003, Selkoe et al. 2006, Burford & Larson 2007, Gonzalez-Wanguemert et al. 2007), and which is likely to generate significant and large F_{IS} values. In the present study, a possible origin of genetic patchiness, involving admixture of seeds from different origins, and a possible low number of trees at the origin of those events of recruitment (bottleneck effect), are also supported by the lack of trends in the evolution of allelic richness

when analyzed in each 5 yr cohort rather than in cumulative age classes (Fig. 3).

These results show a sizable increase in allelic richness during natural recolonization following a catastrophic mortality event. Yet the time for recovery of genetic diversity seems much longer (at least 3 decades) than that for recovery of forest cover and density, which occurred about 2 decades before the present study took place (Hong 1996). Estimates of the genetic recovery rate during the course of recolonization processes are still scarce in the literature. In a few studies on birds (Keller et al. 2001) and marine invertebrates (Barber et al. 2002, Colson & Hughes 2004), genetic variability recovered surprisingly rapidly after local extinction, apparently due to significant and continuous immigration from adjacent healthy populations. Conversely, slow (Sezen et al. 2005) genetic recovery was reported in a tropical tree during regeneration of second-growth forest, apparently due to high variance in reproductive success in the source population. In the case of *Avicennia alba*, without data prior to disturbance nor nearby reference populations of comparable effective size, it is not possible to unambiguously identify the cause of the decline in the rate of genetic recovery of *A. alba* in the Mekong Delta in the mid-1990s. This slower recovery rate may not reflect the achievement of pre-disturbance allelic richness but might instead be due to the complete recovery in population cover/density having been reached years before the present study took place, as competition for space would increase with recovery of forest density. The decline in the rate of increase in allelic richness may reflect a combination of reduced total recruitment and/or the possible prevalence of autochthonous recruitment over that from seeds derived from distant sources as the forest became denser. Moreover, the recent heterozygote deficiency and linkage disequilibrium may be due to recolonization from multiple genetically differentiated sources and/or non-random mating due to a micro-spatial Wahlund effect and/or inbreeding in the recently founded population. Therefore, these results are in agreement with the hypothesis of an increasing importance of autochthonous recruitment that, together with possible inbreeding, may contribute to reduce the rate of genetic recovery. Hence full recovery of the original genetic diversity might only be possible if small-scale patchy disturbance opens new windows of opportunity for allochthonous recruitment.

These results draw attention to the need for a more balanced appraisal of the processes involved in the recovery of ecosystems from disturbance, addressing not only the recovery of the plant communities and associated functions, but also that of the genetic diversity in the ecosystem. Indeed, our results show that genetic recovery can be a significantly longer process than density recovery. The destruction of the Mekong Delta mangrove

forests by Agent Orange is arguably the largest, deliberate, human-driven disturbance yet experienced by any one ecosystem. Recent assessments have revised upwards the impact of Agent Orange on human health in Vietnam (Butler 2003); the results presented here suggest a similarly pervasive effects on the ecosystem. An encouraging result, however, is the suggested ability of *Avicennia alba* to recolonize from external sources despite low propagule dispersal in normal conditions (Duke et al. 1998, Clarke & Kerrigan 2002); this is supported by strong genetic structure at the local scale in congeneric species (Giang et al. 2003, Kado et al. 2004, Arnaud-Haond et al. 2006). This may indicate the existence of density-dependent migration success in this mangrove species, suggesting that estimates of population genetic structure or effective migration obtained in undisturbed conditions may not provide accurate predictions of recolonization potential after local extinction.

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