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Hydrologically contrasting environments induce genetic but not phenotypic differentiation in *Solanum dulcamara*

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Summary

1. In contrasting habitats, locally adapted populations are expected to evolve through directional selection. Hydrological gradients provide a scenario where strong selection forces have led to species segregation in communities along the hydrological niche axes due to specific adaptations to their ambient environment. However, much less is known about the processes enabling species with wide ecological amplitudes, such as *Solanum dulcamara* L., to occupy the contrasting niches at the far ends of the gradient. Here, we investigate the question whether local adaptation is indeed the driving mechanism enabling this species to inhabit contrasting wet and dry habitats.

2. Using molecular analyses and performing a common garden experiment, we examined whether genetic and phenotypic differentiation had occurred between populations originating from flooding- and drought-prone habitat pairs from nine different locations. We also tested whether plants were better adapted to their home site stress.

3. We found a significant genetic variation but hardly any phenotypic differentiation between populations from contrasting habitats for plants grown under optimal conditions. Surprisingly, comparison of the genetic differentiation in neutral markers with that in phenotypic traits using the Q_{ST}-F_{ST} (estimated by Φ_{PT}) approach revealed that the Q_{ST} values were consistently and significantly smaller than the Φ_{PT} values. This is in contrast to the hypothesis that directional selection is an important component enabling *S. dulcamara* to survive under contrasting habitat conditions. In addition, no home site effects were detected for responses to flooding and drought treatments. All plants were characterized by high levels of plasticity for traits associated with flooding and drought tolerance, such as rapid adventitious root formation upon flooding and increased root to shoot ratio in response to drought, irrespective of habitat of origin. Moreover, no trade-offs in such flooding and drought responses were found.

4. *Synthesis*. Our results show that *S. dulcamara* has not evolved locally adapted populations in response to flooding and drought stress, despite genetic differentiation and despite the presumably strong selection gradient. The generally high levels of adaptive plasticity in traits increasing flooding and drought tolerance may be the main mechanism allowing *S. dulcamara* to occupy hydrologically contrasting habitats.

Key-words: amplified fragment length polymorphism, bittersweet, drought, ecological amplitude, flooding, genetic variation, intraspecific variation, phenotypic differentiation, plant-climate interactions, plasticity

Introduction

Directional selection is commonly believed to be the driving force leading to locally adapted genotypes or ecotypes, thereby enabling species to inhabit contrasting habitat types

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(Hall & Willis 2006; Sambatti & Rice 2006; Wright, Stanton & Scherson 2006; Stroup 2015). However, the results from empirical studies conducted on species occurring in such divergent habitats do not always match this prediction (Callahan & Pigliucci 2002; Chen *et al.* 2009; Gimeno *et al.* 2009; Anderson & Geber 2010; Souther, Lechowicz & McGraw 2012). This indicates that the evolution of locally adapted genotypes may depend on the specific ecological settings as well as on other constraints, such as the lack of genetic variation and a high frequency of gene flow (Galloway & Fenster 2000; Anderson & Geber 2010). Adaptive phenotypic plasticity, which enables a genotype to produce multiple phenotypes each adapted to its local environment, has been proposed as an alternative strategy enabling plants to cope with short-term changes in environmental conditions (Pigliucci, Murren & Schlichting 2006; Pratt & Mooney 2013). With habitat conditions becoming less predictable due to increasing climate extremes, the latter mechanism of adaptation may lead to improved performance under these changing conditions as compared to the relatively slow selection processes leading to the evolution of new ecotypes (van Gerven *et al.* 2015). However, such plasticity would typically not be the main mechanism expected in more stable environments.

Hydrology is one of the major factors shaping the vegetation patterns and is expected to be altered by climate change (Silvertown *et al.* 1999; Fraaije *et al.* 2015). Spatial niche segregation has been widely found along hydrological gradients (Silvertown, Araya & Gowing 2015). Flooding and drought, as the two contrasting extremes of the hydrological gradient, impose completely different constraints for growth to plant species, that is oxygen deficiency vs. water shortage (Bailey-Serres & Voesenek 2008; Jaleel *et al.* 2009). Consequently, different phenotypic traits are expected to be selected for by these two stress factors. We investigated for a widespread plant species that occupies both extremes of a hydrological gradient whether ecotype formation explains its wide ecological amplitude, by measuring the genetic variation and morphological responses of populations sampled from the field, in a common garden experiment.

Traits including enhanced shoot elongation, adventitious root development and aerenchyma formation are selected for in flooding-resistant plant species (Visser, Blom & Voesenek 1996; Colmer & Voesenek 2009), whereas traits such as strong regulation of stomatal closure, large and deep root systems and high water-use efficiency are favoured in drought-tolerant species (Jaleel *et al.* 2009). Some of these traits that increase flooding and drought tolerance are mutually exclusive. For instance, drought favours deep rooting capacity (Wasson *et al.* 2012; Lynch 2013), whereas in flooded conditions, plants usually grow their roots very shallowly in the upper soil layer where oxygen might still be available (Baker *et al.* 2001). These contrasting phenotypes in response to the two stress factors indicate that divergent selection for these traits is very likely to occur, reminiscent of the distribution of species with different flooding adaptations over flooding gradients (van Eck *et al.* 2004; Lenssen *et al.* 2004; Voesenek *et al.* 2004). However, contrary to this expectation, our previous work showed little difference in the expression of phenotypic traits and performance between populations of *Solanum dulcamara* L. originating from flooding- and drought-prone habitats of two independent locations when subjected to partial flooding (Visser *et al.* 2015). Therefore, the question arises if ecotype formation is indeed a mechanism that explains the wide distribution of this species across distinctly different habitats. A comparison of a larger number of contrasting habitat pairs is needed to answer this question, as relying on data from a small number of locations carries the risk that local conditions and random genetic effects may conceal general patterns.

Bittersweet (*Solanum dulcamara* L.) is a perennial species that occurs in a broad hydrological range of habitats, from continuously flooded conditions to dry conditions with soil water content reaching levels as low as 3% (w:w; Q. Zhang, unpublished data). We conducted a common garden experiment in a glasshouse with nine population pairs, each representing one wet and one dry habitat collected in the same geographical area. We examined both the genetic and phenotypic differentiation as well as plastic responses to the environment, to investigate the mechanisms enabling *S. dulcamara* to occur in continuously flooded as well as in drought-prone environments. We specifically addressed the following questions: (i) 'Does genetic variation exist between populations originating from wet and dry habitats?'; (ii) 'Do consistent phenotypic differences occur between these local populations when subjected to optimal, common garden conditions?'; (iii) 'Do populations from wet and dry habitats differ in their responses to the environmental stress factors flooding and drought?'; (iv) 'If so, do these local populations show an improved performance when subjected to their home site stress?'

Materials and methods

SPECIES AND SEED COLLECTION

Solanum dulcamara is a perennial Eurasian species within the Solanaceae family. It is outcrossing (Golas *et al.* 2010), but self-compatible (Wright & Barrett 2010) and also capable of vegetative regeneration (Waggy 2009). *Solanum dulcamara* occurs in a broad range of ecologically contrasting habitats, ranging from permanent wetlands to dry costal dunes (Dawood *et al.* 2014; Visser *et al.* 2015).

In autumn 2012 and 2013, seeds of *S. dulcamara* were collected at nine locations along the coast of the Netherlands (Fig. 1). At each location, habitats with contrasting hydrological characteristics were present. A wet population was sampled at the shore of freshwater dune lakes where plants are typically partially submerged throughout the year. A dry population was sampled at dry primary sand dunes well above the seasonally flooded dune slacks, where plants are extremely unlikely to experience flooding. For the dry habitats, the top soil dries out completely in summer; for example in the dry dune habitat in Texel soil moisture content reached values of around $2 \pm 0.7\%$ (w:w; $n = 3$) at the soil surface and $3 \pm 0.6\%$ ($n = 2$) in 45- to 50-cm-deep soil in late spring (Q. Zhang, unpublished results). At each habitat, we collected the seeds from as many different individual plants as possible (at least 10–15 individuals) with a minimum

Fig. 1. Schematic overview of the nine sampling locations along the coast of the Netherlands. At each location, two contrasting habitats, that is freshwater dune lakes and dry primary sand dunes, are present at, on average, <2 km apart.

distance of 5 m between plants to get a representative seed sample covering the genetic variation present in each population. Depending on the habitat of origin, individual plants experienced either only flooding or only drought stress throughout their lifetime. The distance between wet and dry habitats from a given location was 2 km on average and ranged from 0.7 to 4.7 km, which is within the maximum pollination and/or seed dispersal distance (Pasquet *et al.* 2008), leading to a high potential of gene flow between these two habitats in each location. The minimum distance between neighbouring locations was 13.8 km. After collection, the seeds of each population were pooled, cleaned, dried at room temperature and then stored at 4 °C.

PLANT MATERIAL

A hundred and fifty seeds from each of the 18 populations were surface-sterilized with 15% (w:v) trisodium phosphate (Sigma Aldrich Chemie GmbH, Steinheim, Germany) solution for 20 min and then rinsed carefully with tap water. After sterilization, the seeds were sown in seed trays with cells of 3.5 × 3.5 × 4 cm (length × width × depth) filled with moist commercial sowing compost (117.6 mg L^{-1} N, 58. 8 mg L^{-1} P, 125.2 mg L^{-1} K, 4.2 mg L^{-1} Mg, 159.6 mg L^{-1} S, 0.25 mg L^{-1} B, 1 mg L^{-1} Cu, 0.76 mg L^{-1} Fe, 1.3 mg L^{-1} Mn and 0.3 mg L^{-1} Zn; Horticoop substrate, Lentse potgrond & Slingerland potgrond, Cuijk, the Netherlands), covered by plastic and subsequently kept at 4 °C in a cold chamber for 3 weeks to break the dormancy. Thereafter, the seed trays were transferred to the glasshouse for germination for 3 weeks. The seed trays were covered with transparent plastic for 2 weeks to maintain high humidity and stimulate germination. Temperature and humidity in the glasshouse were on average 17.5 ± 0.3 °C (mean ± SD) and 65.5 ± 5.2% (mean ± SD), respectively, during the day and 15.6 ± 0.1 °C (mean ± SD) and 65.1 ± 5.1% (mean ± SD), respectively, during the night. Homogeneously germinated seedlings were transplanted into individual pots of 1.35 L, containing nutrient-poor soil (2.77 mg L^{-1} NO_3^-, 0.05 mg L^{-1} NH_4^+ and 0.06 mg L^{-1} PO_4^{3-}; 70% sand and 30% clay; v:v) supplied with 4 g L^{-1} slow-release fertilizer (Osmocote® Exact Standard, NPK 15-9-12 + 2 MgO + tracing elements, release time 5–6 months, Everris International B.V., Geldermalsen, the Netherlands). Each plant received 60 mL of nutrient solution (2 g L^{-1} Kristalon in tap water, Yara International ASA, Vlaardingen, the Netherlands) 1 week after transplanting to avoid nutrient limitation at the onset of the experiment. Plants were regularly watered in the glasshouse for 5 weeks before the onset of the treatments.

DNA ISOLATION AND AFLP ANALYSIS

Amplified fragment length polymorphism (AFLP) markers were used because of their advantages for assessing genetic differentiation among populations (Mueller & Wolfenbarger 1999; Gaudeul *et al.* 2004). Twenty-four plants from each of the 18 populations were selected to perform the AFLP analysis. To isolate DNA, the CTAB DNA miniprep (Weigel & Glazebrook 2002) was modified as described below. A young leaf of about 2 cm^2 was collected from each plant, frozen in liquid nitrogen and ground in a Mixer Mill MM300 (Retsch GmbH, Haan, Germany) for 30 s at 30 Hz. The frozen leaf tissue was suspended in 500 µL CTAB buffer (0.1 M Tris–HCl pH 7.5, 0.7 M NaCl, 0.01 M EDTA pH 8 and 1% CTAB (w:v)) and incubated at 60 °C for 40 min. A chloroform–isoamyl alcohol extraction was performed by adding 250 µL chloroform–isoamyl alcohol (24:1, v:v), mixing well and centrifuging for 4 min at 151 *g*. Two hundred microlitres of the watery upper phase was added to new collection tubes containing the same volume of isopropanol. After mixing, 10-min incubation at room temperature and centrifugation at 151 *g* for 10 min, the pellet was recovered, washed with 70% ethanol (v:v) and vacuum-dried. The DNA pellet was then dissolved in 200 µL MilliQ water.

The AFLP analysis was performed as described by Peters *et al.* (2001). Three different primer combinations were used: (i) *Eco*RI + AAC (GACTGCGTACCAATTC**AAC**) + *Mse*I + CTG (GATGAGT CCTGAGTAA**CTG**); (ii) *Eco*RI + ACC (GACTGCGTACCAAT TC**ACC**) + *Mse*I + CAA (GATGAGTCCTGAGTAA**CAA**); and (iii) *Eco*RI + ACC (GACTGCGTACCAATTC**AAC**) + *Mse*I + CTT (GAT GAGTCCTGAGTAA**CTT**).

COMMON GARDEN EXPERIMENT

Treatments

For each of the 18 populations, 27 plants were used for the glasshouse experiment. These 27 plants from each population were equally and randomly assigned to control (well-watered), flooding or drought treatments, resulting in nine replicates for each treatment. Plants subjected to flooding treatment were flooded to 10 cm above the soil surface in nine square containers (length × width × height, 98 × 98 × 28 cm) each containing one plant from each of the 18 populations. The soil moisture for control plants and drought-treated plants was kept at 25% and 12% (v:v), respectively, by replenishing the lost amount of water every one to two days. The volumetric water content in the soil was measured with a theta probe (HH2, Moisture Meter version 2, Delta-T Devices LTD, Cambridge, UK). Wilting occurs in this soil-pot system when soil moisture is below 10% (Q. Zhang, personal observation). The treatments lasted for 3 weeks, after

which the plants were harvested. This duration of the experiment was chosen based on the rapid morphological responses of this species to different environmental factors in previous studies, where the consequences of the morphological changes were observed within 2–3 weeks (Visser *et al.* 2015; Q. Zhang, unpublished data).

Measurements

Five extra plants from each population were harvested 1 day before the onset of the treatments to determine the initial plant height (68.0 ± 1.4 cm, $n = 18$ populations) and biomass (2.92 ± 0.08 g, $n = 18$ populations). The youngest fully expanded leaf on the main shoot from each plant was marked with a permanent marker pen 1 week after the onset of the treatments, to indicate which leaves fully developed during the treatments. One day before the final harvest, the leaf above the marked leaf was scanned with a scanner (Epson Expression 11000XL, Seiko Epson Corporation, Nagano, Japan) and the size of the leaf was determined by the projected area calculated with WinRHIZO (Regent Instruments Inc., Québec, QC, Canada). At the final harvest, adventitious roots were counted and harvested separately. The height of the main shoot, the number of side branches and the length and thickness of the internode above the marked leaf were determined. Internode thickness was measured using a digital vernier calliper. Plants were also scored for the presence of flowers at harvest time. No fruit was set at that time. Root systems were carefully washed, and the plants were then divided into roots, stems and leaves and dried to constant weight at 70 °C, after which dry weights were measured.

DATA ANALYSES

AFLP data

Fragment scoring was performed manually from the gel images, and the absence and presence of bands were scored as 0 and 1, respectively. If a band could not be scored unequivocally, a missing value was indicated as −1. For each of the 18 populations, 20–24 individuals were scored and the three AFLP primer combinations resulted in 95 polymorphic AFLP loci: 28 from *Eco*RI+AAC/*Mse*I+CTG, 25 from *Eco*RI+ACC/*Mse*I+CAA and 42 from *Eco*RI+ACC/*Mse*I+CTT. The resulting binary matrix was analysed by a principal coordinate analysis (PCoA) in GENALEX 6.5 (Peakall & Smouse 2012). A two-way ANOVA (type III sum of squares) was conducted on the first coordinate to test for the effects of habitat and location and their interaction (R Development Core Team 2014) using the CAR package (Fox & Weisberg 2011). Two separate nonparametric Kruskal–Wallis tests were conducted due to non-homogeneous variance on the second coordinate to test for the effects of habitat and location; P values were adjusted according to the Bonferroni correction. Since some locations seemed to show specific wet and dry habitat clustering, an additional principal coordinate analysis was performed for each location separately. The derived first and second coordinates were then analysed by separate nonparametric Wilcoxon–Mann–Whitney tests due to non-homogeneous variance to test whether these coordinates differed between habitats within each of the nine locations (Dytham 2011; Ravenscroft, Fridley & Grime 2014) in R. Bonferroni correction was conducted to adjust P values to take multiple comparisons into account. To determine the genetic differentiation between populations, pairwise population differentiation Φ_{PT} values were computed from analysis of molecular variance (AMOVA) using 999 permutations. A Mantel test, as implemented in GENAIEX 6.5, was performed using genetic and geographical distances to test for isolation by distance (Mantel 1967).

Phenotypic data

All analyses were conducted in R using the LME4 (Bates *et al.* 2015) and CAR packages. Data were analysed using a split-plot analysis of variance (ANOVA, type III sum of squares) to test the main effects of treatment and habitat and their interaction. The split-plot model was built according to Crawley (2005). In this model, habitat was nested within the random factor location, and the habitat effect was tested over the mean sum of squares of the 'habitat × location' interaction error. The main effects and their interaction on the presence of flowers were analysed by conducting a logistic regression. Since branch number was count data, the main effects of treatment and habitat and their interaction on this variable were analysed by conducting a Poisson regression. Separate one-way ANOVAs, logistic regressions and Poisson regressions comparing plants from dry and wet habitats under each treatment were then performed to determine the direction of the interaction between treatment and habitat on root to shoot ratio, root biomass, branch number and the presence of flowers. The habitat effect on number and biomass of adventitious roots was analysed with one-way ANOVA (type III sum of squares) where habitat was treated as main effect and location as random effect. Total root biomass was reciprocally transformed, and leaf size was transformed by a power transformation where the constant e was raised to the power of the leaf size data, and the other data were log10-transformed if necessary to increase the homogeneity of the variances.

To test whether a consistent phenotypic difference occurred between habitats and among populations under common garden conditions, a principal component analysis (PCA) using the built-in *prcomp* function was performed for all nine locations. A subset of the traits was used for the analyses, including total biomass and seven phenotypic traits, that is root to shoot ratio, stem height, internode length, stem thickness, branch number, leaf size and specific leaf area. Total biomass was included in the PCA as a substitute of growth rate since the initial total biomass was similar across populations. Care was taken to avoid highly correlated or interdependent traits; for example, we did not include root or shoot biomass separately, because we already had included total biomass and root to shoot ratio in the analyses. We performed a two-way ANOVA (type III sum of squares) on the PCA scores of both components 1 and 2 to test the overall effects of habitat and location and their interaction. As there was a significant interaction between habitat and location, and also because we were interested in the habitat difference in each location, the same PCA was also performed for each location. Student's t-test was then conducted on the PCA scores of components 1 and 2 between dry and wet habitats within each location (Dytham 2011; Ravenscroft, Fridley & Grime 2014). To test whether a different general response to stress conditions existed between habitats over the nine locations, another PCA was conducted on the relative stress responses of the populations calculated from the total biomass and the same seven phenotypic traits used in the separate PCAs. The response to stress including both flooding response and drought response was calculated as $100 \times (\text{Mean}_{\text{flooding/drought}} - \text{Mean}_{\text{control}})/\text{Mean}_{\text{control}}$; a positive or a negative value indicates an increased or decreased trait value in response to stress, respectively. To test the main effects of habitat and treatment and their interaction, a two-way ANOVA (type III sum of squares) was conducted on the PCA scores of components 1 and 2. Due to our interest in the habitat effect under

each treatment, separate PCAs for each treatment were conducted and their PCA scores of components 1 and 2 were compared, respectively, between wet and dry habitats for each treatment using separate Student's *t*-tests (Dytham 2011; Ravenscroft, Fridley & Grime 2014). Bonferroni correction was conducted to adjust *P* values to take multiple tests into account for all the separate tests for the nine locations and three treatments.

Q_{ST}-F_{ST} comparison

A Q_{ST}-F_{ST} comparison was made to detect local adaptation and to disentangle the effects of directional selection from that of other evolutionary processes, such as genetic drift. F_{ST} is a measure of genetic differentiation among populations estimated by neutral molecular markers (Whitlock 2008). We used Φ_{PT} to estimate F_{ST} as Φ_{PT} is an analogue and a good estimator of F_{ST} (Steinger *et al.* 2002; Gruenthal, Acheson & Burton 2007; Le Roux *et al.* 2014). Φ_{PT} was calculated by subjecting the AFLP data to an AMOVA in GENAIEX 6.5 as described in the section 'AFLP data' of data analyses. For Q_{ST}, we first estimated the within- and between-habitat variance in total biomass and the other seven phenotypic traits used in the PCA in a random-effect ANOVA model using LME4 package in R (R Development Core Team 2014), where habitat was treated as random effect (Steinger *et al.* 2002). These within- and between-habitat variances in total biomass and quantitative traits were then used to calculate Q_{ST}, which is a measure of the genetic differentiation among populations for quantitative traits (Whitlock 2008), with the equation $Q_{ST} = \sigma^2_{GB}/(\sigma^2_{GB} + 2\sigma^2_{GW})$, where σ^2_{GB} is the between-habitat variance and σ^2_{GW} is the within-habitat variance (Leinonen *et al.* 2013). The average Q_{ST} for total biomass and the other seven phenotypic traits were compared with the Φ_{PT} for AFLP markers for all the nine locations using a paired Student's *t*-test in R (R Development Core Team 2014).

Results

GENETIC DIFFERENTIATION WAS PRESENT BETWEEN HABITATS

Populations originating from different habitats and locations differed significantly in both the first and second PCoA coordinates, and a significant interaction between habitat and location existed as well (Appendix Table S1 in Supporting Information). Among the nine locations, the three North Sea islands (Schiermonnikoog, Ameland and Texel) together with Callantsoog and Castricum were separated from the other, more southern locations (Fig. S1).

Within each location, the first principal component coordinate of plants originating from dry and wet habitats differed significantly (Fig. 2). Among these nine locations, Schiermonnikoog, Zandvoort and Callantsoog were the most genetically separated population pairs according to their relatively high ΦPT values (0.288–0.394; Fig. 2), whereas the population pairs in Ameland, Meijendel and Goeree were the least separated (Φ_{PT} values 0.063–0.142; Fig. 2). The second principal component coordinates of plants originating from dry and wet habitats did not differ for any of the nine population pairs (Fig. 2). To investigate whether the different levels of genetic variation could be explained by the geographical distance among populations, a Mantel test was performed between the genetic distance and the geographical distance for dry and wet populations within locations. Within location, a greater geographical distance between the two populations did not translate into a larger genetic distance (Fig. 3a), which was in contrast to the clear effect of geographical distance on molecular population differentiation at the larger, that is location, scale (Fig. 3b).

FLOODING AND DROUGHT STRESS HAD SIGNIFICANT EFFECTS ON PLANT GROWTH AND PHENOTYPE

Flooding and drought significantly suppressed the plant growth in terms of reduced biomass accumulation compared to well-watered control conditions (Table 1, Fig. 4a). Plants also displayed different traits in response to drought and flooding, resulting in phenotypes with higher root to shoot ratio under drought conditions and adventitious root formation in flooded plants, but not in plants in the control and drought treatments (Fig. 4b, k, l). In addition, plants grew shorter and produced thinner main stems under stress than when growing under control conditions (Table 1, Fig. 4d, f). Flooding also significantly decreased specific leaf area (Table 1, Fig. 4i).

PLANTS FROM DRY AND WET HABITATS DID NOT DIFFER IN RESPONSE TO TREATMENTS

Plants originating from dry and wet habitats showed some minor differences in phenotypic traits, such as root biomass, root to shoot ratio and branch number (Fig. 4b, k, l, Table 1). Across the three treatments, plants originating from dry habitats had significantly higher root biomass and root to shoot ratio and produced more side branches than those from wet habitats (Fig. 4b, k, l, Table 1). Under control conditions, plants originating from dry habitats tended to invest more into root growth than those from wet habitats (Fig. 4c). When flooded, plants originating from dry habitats had shorter internodes than those from wet habitats (Fig. 4g). Habitat had no effect on flowering ratio of plants under control conditions; however, under flooding and drought conditions, plants originating from dry habitats had a higher flowering ratio than plants from wet habitats (Fig. 4j). This is due to different responses in flowering time of plants to the local and foreign stress. Plants from dry habitats did not respond to the foreign flooding stress, but responded to their local drought stress with earlier flowering. Plants from wet habitats, on the contrary, did not respond to the foreign drought stress, but delayed flowering when flooded (Fig. 4j). Remarkably, plants originating from either wet or dry habitats responded similarly, rather than differently, to the flooding and drought treatments for most morphological traits (Table 1, Fig. 4).

CONSISTENT PHENOTYPIC DIFFERENCES EXISTED BETWEEN HABITATS UNDER COMMON GARDEN CONDITIONS RATHER THAN UNDER STRESS CONDITIONS

To test whether consistent phenotypic differences over a range of phenotypic traits existed between habitats under

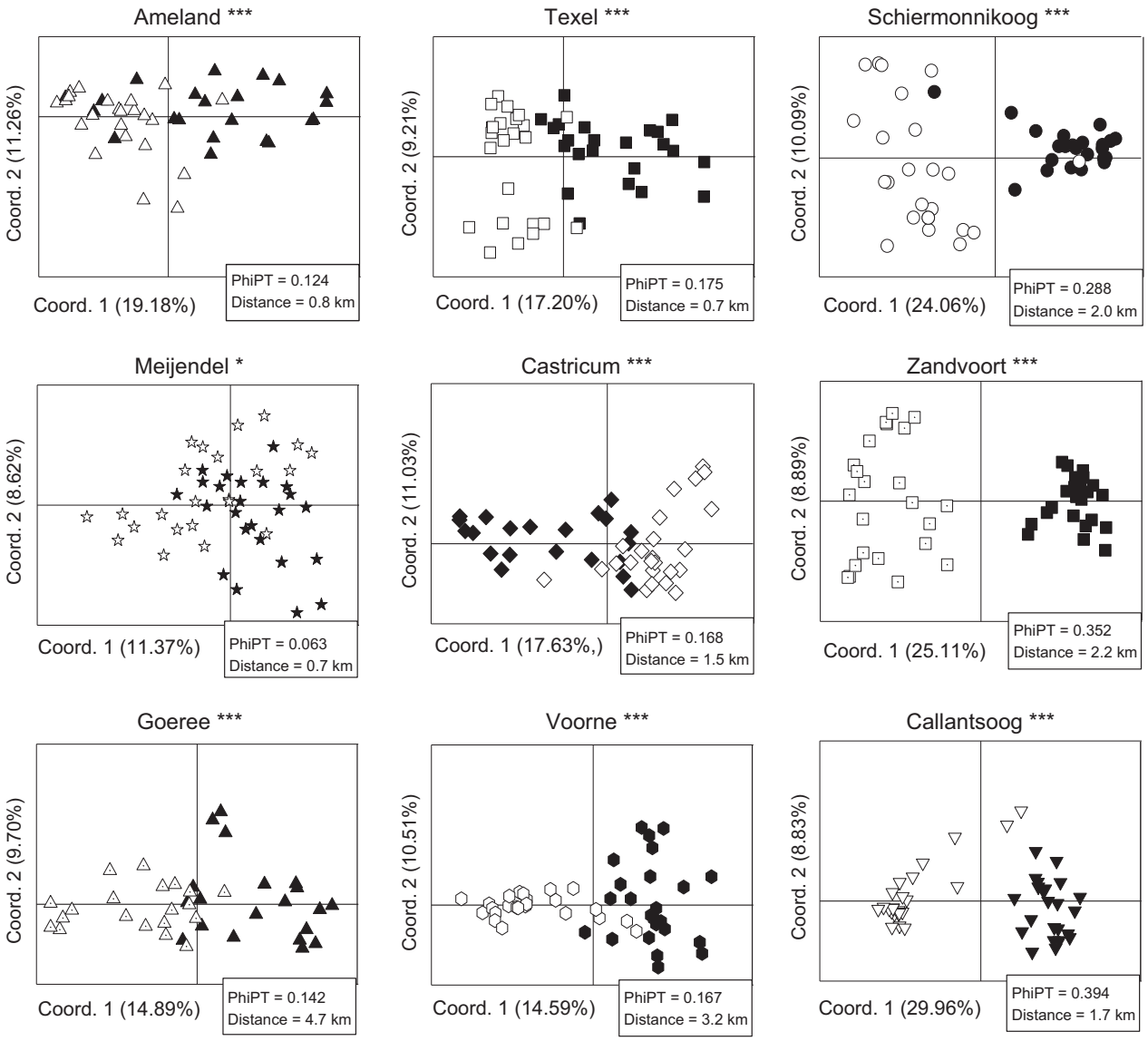

Fig. 2. Diagrams presenting the main axes of a principal coordinate analysis (PCoA) on the genetic distance matrixes of plants originating from dry (open symbols) and wet (shaded symbols) habitats within each of the nine locations along the coast of the Netherlands, based on 95 AFLP markers. Significant results of the nonparametric Wilcoxon–Mann–Whitney tests comparing the PCoA scores (Coord. 1; no significant results for Coord. 2) between habitats per location are indicated with asterisks (**)). The significance levels are adjusted into ***$P < 0.0001$, **$0.0001 < P < 0.0011$ and *$P < 0.0056$ to take multiple comparisons into account. Each symbol represents an individual plant.

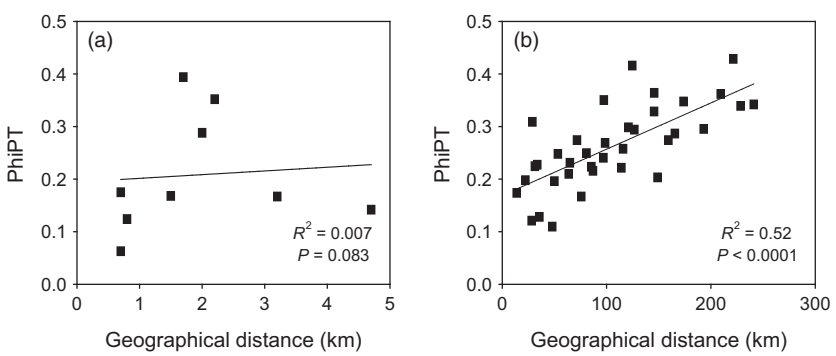

Fig. 3. Results from a Mantel test analysing the relationship between the geographical distance and the genetic differentiation (Φ_{PT} (PhiPT)) (a) of plants between the wet and dry habitats of each location and (b) between any two out of the nine locations. The R^2 (the proportion of explained variability) and the significance of the correlation are displayed in each graph.

Table 1. The *F*-values and their significance analysed by means of split-plot ANOVA for the main effect of treatment, habitat and their interactions on total biomass, root biomass, root to shoot ratio, SLA, stem height, internode length and thickness of the internode and chi-squares and their significance of logistic regression on flowering ratio and Poisson regression on branch number for the effect of treatment and habitat and their interaction. Habitat effect was tested over the mean sum of square of the 'habitat × location' interaction. Adventitious roots were included in root biomass and the calculation of root to shoot ratio

	F-values/Chi-squares										
	d.f.	Total biomass	Root biomass	Root/shoot ratio	Leaf size	SLA	Stem height	Branch number	Internode length	Stem thickness	Flowering ratio
Treatment (T)	2	**60.485*** **	**32.290*** **	**46.212*** **	0.833^{ns}	**51.829*** **	**70.347*** **	1.074^{ns}	**46.538*** **	**58.152*** **	**18.591*** **
Habitat (H)	1	1.017^{ns}	**4.512***	**6.904***	1.482^{ns}	0.300^{ns}	1.921^{ns}	**6.847****	1.366^{ns}	0.015^{ns}	0.076^{ns}
T × H	2	1.209^{ns}	$2.889^{\$}$	1.740^{ns}	1.801^{ns}	0.810^{ns}	0.300^{ns}	1.031^{ns}	$2.700^{\$}$	0.676^{ns}	**6.756***

The significance levels are indicated as: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, $^{\$}0.05 < P < 0.1$, $^{ns}P > 0.1$. Significant values ($P < 0.05$) are in boldface.

common garden conditions, a principal component analysis (PCA) was performed on total biomass and seven phenotypic traits (root to shoot ratio, stem height, internode length, stem thickness, branch number, leaf size and specific leaf area) of plants in the control treatment. The two-way ANOVA on the PCA scores showed that location had a significant effect and habitat a marginally significant effect on the first component (Table S3). Neither habitat nor location affected the second component (Table S3). Separate comparisons of the PCA scores for each population pair revealed that for none of the nine population pairs, habitats were significantly separated along the first or second axis based on the phenotypic data (Fig. 5), with only marginally significant effects of habitat for Schiermonnikoog along the second axis and a marginally significant separation along the first axis for Meijendel (Fig. 5). Interestingly, only Schiermonnikoog was characterized by strong molecular differentiation as well, whereas Meijendel was among the least separated population pairs according to the AFLP data (Fig. 2). When the genetic differentiation for total biomass and quantitative traits (Q_{ST}) was compared with that for the AFLP markers (Φ_{PT}) between the wet and dry habitats within each location, we found significantly and consistently smaller values of Q_{ST} (range: 0.012–0.104; Table S2) than those of Φ_{PT} (range: 0.063–0.394; Table S4) ($t = -3.69$, $P = 0.006$). In addition to the lack of substantial differences for trait values under control conditions, habitat of origin did not affect the response of those traits to drought or flooding either (Fig. 6, Table S5).

Discussion

In line with our hypothesis that strongly contrasting habitats would lead to segregating populations, clear genetic differentiation occurred between populations of *Solanum dulcamara* originating from wet and dry habitats, indicating that there was a potential for divergent selection. Surprisingly, however, hardly any phenotypic differentiation among plants from different habitats occurred under common garden conditions. These results indicate that flooding and drought stress did not impose consistent directional selection pressures across locations and traits. We therefore did not find evidence for local adaptation explaining the wide ecological soil moisture range *S. dulcamara* can cover, which was also supported by the relatively lower between-habitat differentiation in terms of phenotypic (Q_{ST}) as compared to molecular (Φ_{PT}) values. In addition, the result $Q_{ST} < F_{ST}$ (estimated by Φ_{PT}) also suggests that the observed lack of differentiation in total biomass and phenotypic traits between habitats was not due to genetic drift ($Q_{ST} = F_{ST}$) (Leinonen *et al.* 2013). Moreover, no relative advantage in terms of increased performance was found in plants subjected to treatments reflecting their respective home site stress conditions, that is flooding and drought. Apparently, despite the clear genetic differentiation, evolution of locally adapted populations in response to flooding and drought stress had not occurred in *S. dulcamara*.

FLOODING AND DROUGHT HAVE NOT DRIVEN DIRECTIONAL SELECTION IN *S. DULCAMARA*

Flooding and drought are strong selection forces that have been shown to affect the distribution and abundance of plant species, leading to discriminating zonation patterns in natural habitats (Blom *et al.* 1994; van Eck *et al.* 2006; Silvertown, Araya & Gowing 2015). Locally adapted genotypes can be even selected for in species subjected to fine-grained spatial variation along flooding or drought gradients (Lenssen *et al.* 2004; Franks 2011). Therefore, more pronounced local differentiation may be expected when two divergent selection forces act on geographically separated (sub)populations of the same species (Heschel *et al.* 2002; Hereford 2009). *Solanum dulcamara* populations display very different phenotypes in their natural habitats when comparing plants growing in contrasting hydrological conditions (D'Agostino *et al.* 2013). In wet habitat, plants are characterized by long, hardly branched stems and relatively large leaves, while in dry habitat plants are characterized by short, highly branched stems and small leaves. In addition, locally adapted genotypes of this species

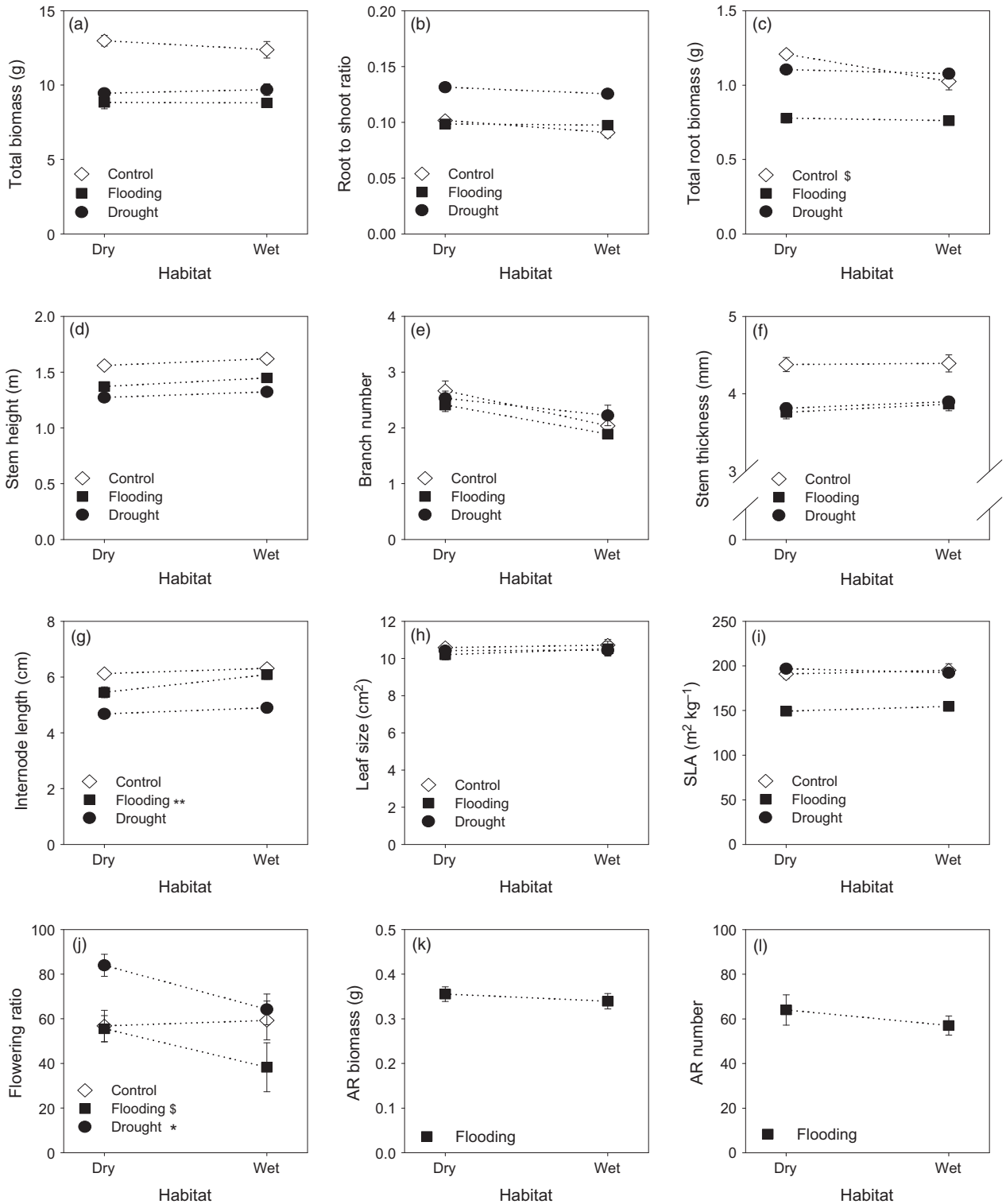

Fig. 4. Phenotypic responses of morphological traits in populations originating from either dry or wet habitats in control, flooding and drought treatments. Adventitious roots were included in root biomass and the calculation of root to shoot ratio. Means are averages of nine populations; error bars indicate SE. (Marginally) significant differences between habitats in each treatment are indicated as: $^{**}P < 0.003$, $^{*}0.003 < P < 0.017$, $^{\$}0.017 < P < 0.03$; P values were adjusted according to the Bonferroni correction.

have previously been revealed in response to light availability (Gauhl 1976, 1979). We therefore expected to detect local adaptation in this species in response to water availability. However, *S. dulcamara* showed little evidence for adaptive population divergence in response to flooding and drought, despite the clear genetic differentiation between the two habitat types. Below, we will explore the potential explanations for this result.

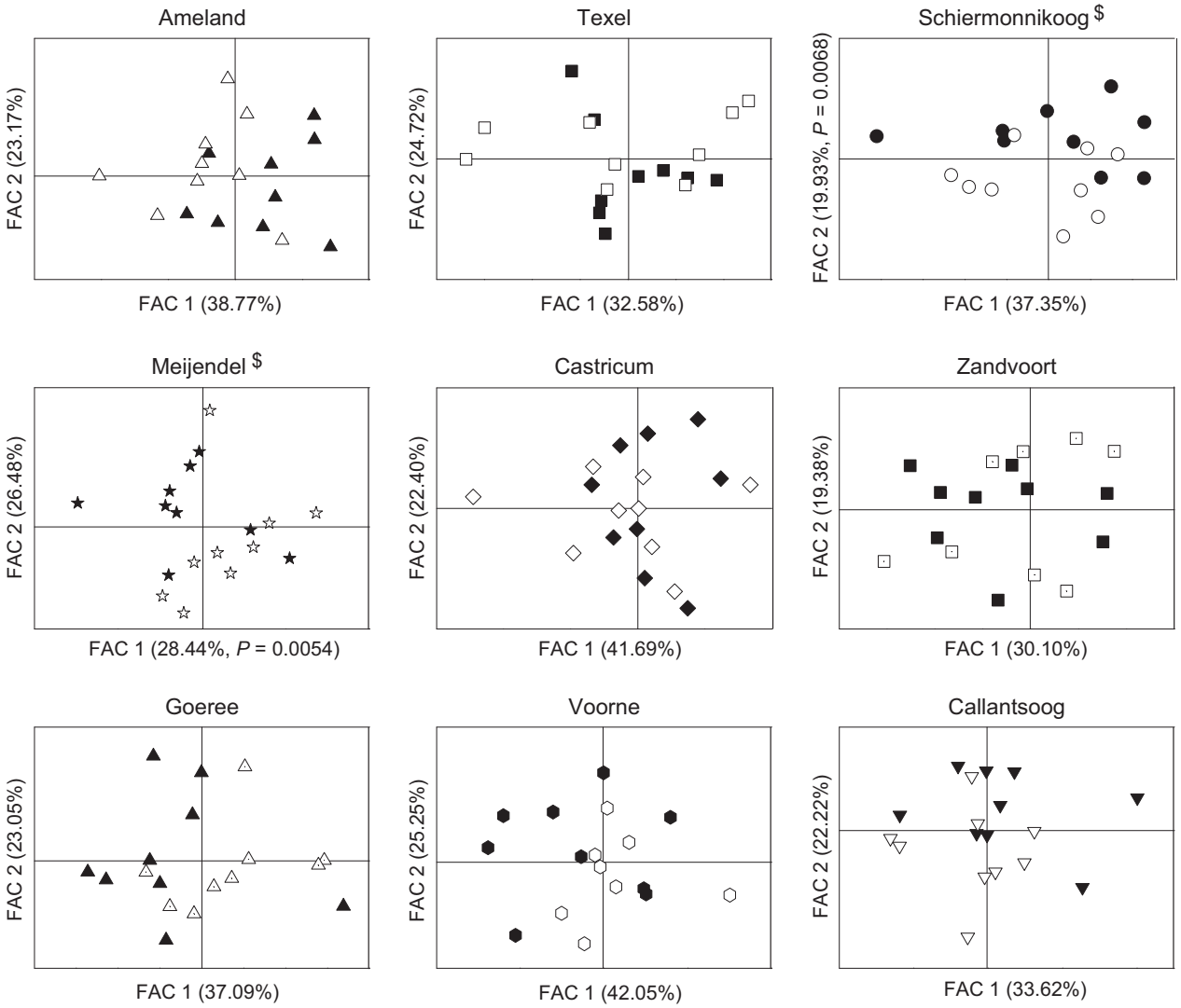

Fig. 5. Diagrams presenting the main axes of a principal component analysis (PCA) of plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in control treatment. The PCA scores (factors 1, 2) are derived from total biomass, root to shoot ratio, stem height, branch number, stem thickness, internode length, leaf size and SLA of individual plants. *P* values were adjusted according to the Bonferroni method; marginally significant results ($P < 0.01$) of the Student's *t*-test comparing the PCA scores (factors 1, 2) between habitats per location are indicated with *P* values in the X/*Y*-axis and with $ in the title after the location name. Each symbol represents an individual plant.

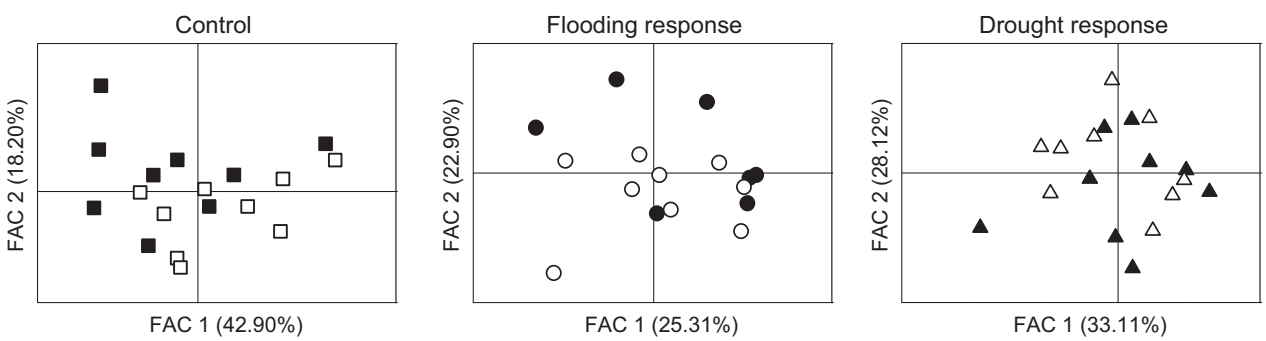

Fig. 6. Principal component analysis (PCA) of the plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in flooding and drought treatments. The PCA scores (factors 1, 2) for control are derived from the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA of a population. The PCA scores (factors 1, 2) for flooding response and drought response are derived from the responses of the above eight variables to flooding and drought. Each symbol represents a dry or wet population from one location.

Lack of genetic variation and homogenizing processes such as gene flow have been argued to counteract adaptive natural selection (Galloway & Fenster 2000; Kawecki & Ebert 2004; Anderson & Geber 2010). However, the former reason does not apply to *S. dulcamara* in our study because of the relatively larger genetic variation as revealed by the AFLP measurements compared to the low differentiation of the phenotypic data. Moreover, despite the high potential for gene flow between different habitats through pollen and seed dispersal by bumblebees and birds, respectively, which are the main pollen and seed dispersal agents for this species (Pasquet *et al.* 2008; Waggy 2009; Golas *et al.* 2010), the observed genetic differentiation between populations within one location suggests gene flow among populations to be limited. Limited pollen flow may be explained by the composition of the local insect pollinator community, which can be very different due to the contrasting environmental conditions and plant species composition in the dry and wet habitats (Lobo, Quesada & Stoner 2005). If the activity range of these insect pollinators is limited to a specific local habitat, exchange of genes between habitats is not likely to occur, leading to genetic divergence. This provides a prerequisite for directional selection to take place in the two contrasting habitats (Kawecki & Ebert 2004). Similarly, berries of *S. dulcamara* can be eaten and distributed by birds, but bird species frequenting marshes and dune lakes may not be the ones commonly found in the dry dunes. Therefore, a high level of gene flow does not seem a likely explanation for the apparent absence of locally adapted populations in *S. dulcamara*.

Local adaptation also depends on the strength of the divergent selection (Kawecki & Ebert 2004; Leimu & Fischer 2008). When the selection pressures are either mild or when intense temporal fluctuation exists, local adaptation is unlikely to occur (Kawecki & Ebert 2004; Leimu & Fischer 2008). In our study, however, there is no doubt that the flooding and drought stress acting on *S. dulcamara* in their local habitats is strong enough for divergent selection to occur, as in other species ecotype formation has occurred even along a much less pronounced hydrological gradient and at much smaller distances (Lenssen *et al.* 2004; Kooyers *et al.* 2015). Moreover, wet and dry *S. dulcamara* populations co-occurred with a completely different set of species in their respective natural habitats (i.e. marsh and reed vegetation, such as *Phragmites australis*, *Epilobium hirsutum* and *Lythrum salicaria*, vs. dry dune vegetation, such as *Ammophila arenaria, Oenothera spec.*, and *Hippophae rhamnoides*; personal observation). Seasonal variation might counteract local adaptation, and especially, the frequency and severity of flooding may fluctuate. To minimize this potential effect, we collected the seeds only from populations typically experiencing flooding throughout the year, located along the border of dune lakes that rarely dry up even at the end of summer. Therefore, environmental fluctuations or weak directional selection pressures are an unlikely driving force leading to the limited variation in response to wet and dry conditions among the plants from the two types of habitats.

Although the difference in water availability was the most prominent environmental factor differentiating these habitats, we cannot rule out that other environmental factors have also played the role and have led to phenotypic changes that we have not measured. In addition, we did not include the reproductive stage in this experiment. Differences in flowering time in plants subjected to different conditions and different plasticity in flowering phenology in plants originating from different habitats may have contributed to local differentiation and may have led to genetic differentiation. In order to test this hypothesis, a long-term experiment covering the reproductive stage of plants until seed set accompanied by additional measurements of phenology under natural conditions would be required.

PHENOTYPIC PLASTICITY MAY HAVE REPLACED LOCAL ADAPTATION

Our results clearly show that ecotypic differentiation for flooding and drought stress did not occur in our study, and populations from wet and dry habitats showed very similar plastic responses to flooding and drought. For instance, irrespective of habitat of origin, all plants produced numerous adventitious roots, a common flooding acclimation response facilitating gas exchange of flooded plants (Sauter 2013), thus allowing water and nutrient uptake to continue (Jackson 1955; Herzog *et al.* 2015). Adventitious roots have also been shown to positively correlate with plant biomass development of *S. dulcamara* in a previous study (Zhang *et al.* 2015). In this study, the fact that the adventitious rooting ability did not differ between populations from dry and wet habitats is quite surprising and suggests little differential selection among habitat types on this trait. Similarly, drought also induced changes in plant morphology, such as an increased relative investment in the root system, a response typically leading to increased water uptake potential (Gonzalez *et al.* 2013). Lack of difference in such a trait between the two contrasting habitats reinforces the interpretation that there is no apparent trade-off in the ability of plants to adapt either to drought or to flooding. Interestingly, the difference in flowering time in response to flooding and drought suggests that local adaptation in this trait may result in modified plasticity rather than trait means. Altering flowering time in response to flooding and drought stress may result in different flowering phenology in the local plant populations. Such differences in phenology may well contribute to the genetic differentiation at small spatial scales found in our study.

This lack of differences in the expression of inducible flooding- and drought-adaptive traits between populations from contrasting habitats may explain why plants did not show an advantage in terms of lower reduction in performance if subjected to their home site stress. Apparently, all populations were able to maintain sufficient phenotypic plasticity to cope with both contrasting stresses, which raises the question why the ability to respond plastically to a foreign, thus hardly ever occurring, stress had not been selected against. This is in contrast to the well-described trade-offs

between flooding and drought tolerance among species (Araya *et al.* 2011; Silvertown, Araya & Gowing 2015). Possibly, the costs of maintaining this 'redundant' plasticity are not very high in this species (Weijschedé *et al.* 2006; Chen *et al.* 2011). Still, it is surprising that plants, irrespective of habitat, maintained equal level of plasticity in these traits, and irrespective of the opposite requirement on rooting patterns under flooded and drought conditions (shallow vs. deep rooting, respectively). This indicates that there is no developmentally fixed trade-off between flooding- and drought-induced rooting patterns and that selection had apparently favoured high versatility in this trait above developmental fixation (Silvertown *et al.* 1999; Silvertown, Araya & Gowing 2015). The mechanism underlying this lack of trade-off in flooding- and drought-induced rooting patterns needs further investigation.

In line with our results, it has recently been argued that a high level of phenotypic plasticity is the major strategy enabling the marsh grass species *Spartina alterniflora* to spread over 19° latitude along the east coast of China across different soil moisture conditions, since the different phenotypes found in the field disappeared when plants were grown in a common garden (Liu *et al.* 2016). In our study, phenotypic plasticity even enables *S. dulcamara* to colonize habitats at the opposite ends of a soil moisture gradient. It remains an intriguing unsolved question why this species is able to cover such a wide ecological amplitude, without any apparent specific adaptation to the local environment, while most other species are much more confined to a smaller range in the hydrological gradient (Voesenek *et al.* 2004; Moeslund *et al.* 2013). Further study of the characteristics that make *S. dulcamara* such a unique example has a potential to reveal mechanisms increasing our understanding on plant adaptation to different hydrological conditions.

Conclusions

Our results show that *Solanum dulcamara* has not evolved locally adapted populations in response to flooding and drought stress, despite the presumably strong selection gradient. Our results suggest that high levels of adaptive plasticity in the responsive traits in this species, regardless of habitat of origin, is an important mechanism allowing *S. dulcamara* to occur over a wide hydrological gradient. Such high levels of plasticity allowing plants to rapidly respond to environmental changes will most likely enable *S. dulcamara* to be resistant to and potentially even to expand its range in a changing climate. Our results also indicate that existing genetic differentiation does not necessarily predict the differences in phenotypic responses to the environmental conditions, and vice versa.

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Data accessibility

Data from this paper can be accessed through the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.4ht08 (Zhang *et al.* 2016).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Results from a principal coordinate analysis (PCoA) based on the genetic distance matrix of all 18 *S. dulcamara* populations based on 95 AFLP markers.

Fig. S2. Diagrams presenting the main axes of a principal component analysis (PCA) of plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in control treatment.

Table S1. The *F*-values and their significance analysed by means of two-way ANOVA for the effects of habitat and location and their interaction on the first coordinate (Coord. 1) generated from the PCoA analysis on all the nine population pairs, and Chi-squares and their significance of Kruskal–Wallis tests for the effects of habitat and location on the second coordinate (Coord. 2).

Table S2. The genetic differentiation Φ_{PT} values (below diagonal) and the geographical distance (above diagonal) between the 18 populations of *S. dulcamara*.

Table S3. The *F*-values and their significance analysed by means of two-way ANOVA for the effects of habitat and location and their interaction on the first (FAC 1) and second (FAC 2) component scores generated from the the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA through the PCA analysis on all the nine population pairs under control conditions.

Table S4. Genetic differentiation for AFLP markers (Φ_{PT}) and quantitative traits (Q_{ST}) between population pairs of *S. dulcamara* across nine locations.

Table S5. The *F*-values and their significance analysed by means of two-way ANOVA for the effects of habitat and treatment and their interaction on the first (FAC 1) and second (FAC 2) component scores generated from the responses of the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA of a population to flooding and drought through a PCA analysis on all the nine population pairs.