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Genetic and Morphological Variation of Belgian *Cyperus esculentus* L. Clonal Populations and Their Significance for Integrated Management

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Abstract: *Cyperus esculentus* is an invasive troublesome neophyte in many arable crops across the globe. Analysis of the genetic and morphological profile of local *C. esculentus* clonal populations may be useful in explaining differential herbicide sensitivity found among distinct clonal populations and spatial distribution patterns. In this study, 35 Belgian *C. esculentus* clonal populations, evenly spread across the entire infestation area (30,689 km^2) and covering a great diversity of farm and soil types, and hydrological and environmental conditions, were genetically characterized using amplified fragment length polymorphism (AFLP) profiling. These clonal populations were also grouped into different morphological clusters using data from shoot, tuber, and inflorescence number, fresh tuber biomass, individual fresh tuber weight, and number of germinable seeds collected in three consecutive years. Of the 271 AFLP markers, 207 were polymorphic. The gene diversity among clonal populations was 0.331 and three genetically distinct clusters were identified. Depending on observation year, clonal populations were grouped in four to five morphologically distinct clusters that closely aligned with the genetic clusters. The genetically distinct clusters differed in their geographical distribution pattern and range as well as in their morphological characteristics. Clonal populations belonging to clusters with broad distribution ranges produced numerous viable seeds. Clusters with clonal populations that produced large tubers were less widespread than the cluster with clonal populations producing many small tubers. The results suggest that tuber size, tuber number, and fecundity may all play an important role in the spread of *C. esculentus*. Morphotyping may be very useful in designing effective preventive and curative *C. esculentus* management strategies.

Keywords: AFLP; phenotypic plasticity; fecundity; tuber production; yellow nutsedge; local spread; clustering

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1. Introduction

Cyperus esculentus L. (yellow nutsedge) is a serious worldwide threat to agriculture as it is one of the most invasive plants in the world [1]. It belongs to the second largest genus, *Cyperus*, in the *Cyperaceae* family [2]. There is no general agreement on the subdivision of *C. esculentus* into different botanical varieties due to the lack of strong and non-overlapping diagnostic features. Boeckeler [3] was the first to study the intraspecific taxonomy of *C. esculentus* using different cultivated and American forms. The species was divided in a cultivated (var. *sativus* Boeckeler) and an unspecified wild variety by Ascherson and Graebner [4]. Kükenthal [5] proposed a division into seven weedy varieties based on characters of the inflorescence only: var. *esculentus*, var. *cyclolepis* Boeck. ex Kük, var. *nervoso-striatus* (Turrill) Kük, var. *macrostachyus* Boeckeler, var. *sprucei* Clarke, var. *leptostachyus* Boeckeler, and var. *heermannii* (Buckley) Britton. Schippers et al. [6] reviewed this division and retained four wild varieties (vars. *Esculentus*, *leptostachyus*, *macrostachyus*,

and *heermannii*) and a cultivated variety (var. *sativus*) based on 17 geometric morphometrics of generative plant parts, mainly size and shape of floral parts. These varieties have definite origins and geographical distributions. The three additional wild varieties, previously defined by Kükenthal [5], were no longer supported due to lack of evidence found in the work of Schippers et al. [6]. Recent molecular studies by De Castro et al. [7] do not support the abovementioned taxonomic varieties. Therefore, the subdivision of C. esculentus into finer taxonomic categories is not accepted by several researchers [8].

The invasive success of *C. esculentus* is attributed to five main plant characteristics: rapid growth, prolific vegetative reproduction, high competitive ability for light, water, and nutrients, tolerance to different control measures, and plasticity in different environments [9]. Plasticity in *C. esculentus* was first mentioned by Kükenthal [5] whose study populations showed considerable adaptation to local environmental conditions. In Europe, it is mostly found in western and southern Europe as well as in parts of central Europe [10]. In Belgium, *C. esculentus* is found as a weed in almost all arable crops grown in rotation with maize (*Zea mays* L.) and it has infested over 50,000 ha of cropland (S De Ryck pers. comm.), mainly in the northern part of the country (i.e., Flanders and neighbouring northern part of Wallonia) [11] where 88% of Belgian maize acreage is grown [12] and winter conditions are mild (average day and night time temperatures during winter of 6.6 and 1.7 °C, respectively).

Analysing and understanding the genetic and morphological structure of weeds is useful for their control as these factors may affect the efficacy of certain control methods [13]. It also aids in developing biological and chemical control practices [14,15], and in determining the origin and the spread of invasive species [16]. Some studies have investigated the physiological, phenological, and genetic variations among *C. esculentus* populations. Ter Borg et al. [17] described intraspecies variation in morphology and ecology. Li et al. [18] found considerable variation in 20 quantitative traits among 5 Japanese *C. esculentus* clones and reported important levels of phenotypic plasticity in response to water availability. De Cauwer et al. [19] reported significant differences in shoot number, tuber dry biomass, tuber number, individual tuber dry weight, inflorescence number, and capacity to set viable seeds (achenes) among 25 Belgian *C. esculentus* clones. Horak et al. [20] found, in an isoenzyme analysis, relatively low genetic diversity with most of the diversity occurring as differences among individuals within populations as opposed to differences between populations. Holt [21] found considerable genetic variation in quantitative traits. According to Holt, factors such as multiple introductions, habitat heterogeneity, large population size, outbreeding, isolation, polyploidy, and hybridization may all have contributed to these high levels of genetic diversity. Dodet et al. [22] found a low level of genetic diversity (0.14) in southwest France (Haute Lande), indicative for small and isolated populations. De Castro et al. [7] found considerable genetic variation of nuclear versus chloroplast DNA markers (27 ribotypes vs. 6 haplotypes) with clear geographic segregation within the nuclear markers.

As suggested by Mulligan and Junkins [23], interclonal phenotypic and genetic variability of *C. esculentus* could possibly explain differential responses of *C. esculentus* clones to chemical control measures. De Cauwer et al. [19] found large differences in sensitivity to glyphosate, halosulfuron, and dimethenamid-P among morphologically distinct *C. esculentus* clones with up to 8.3-fold differences in doses required for 90% control of aboveground dry biomass. In a study by De Ryck et al. [24], clones showed up to 74 percentage point differences in chemical control of tuber numbers. Clones with a higher median tuber fresh weight were generally (five out of nine strategies) less controlled.

We hypothesised the following: (H1) Belgian *C. esculentus* clonal populations can be grouped into distinct genetic and morphological clusters, (H2) genetic clusters align with morphological clusters and have distinct morphological profiles, and (H3) morphological typing of the genetic clusters aids in understanding geographical distribution patterns and rate of spread, and in explaining between-field variation in control efficacy of *C. esculentus* measures reported by farmers and weed scientists. Hereto, we clustered Belgian

clonal populations according either to their morphotype or to their genotype, studied how the morphological clustering was reflected in the genetic clustering and vice versa, and studied their geographical distribution patterns. Although this study focuses on Belgian *C. esculentus* clonal populations, the information presented hereafter is applicable to other regions and will be of wide interest to farmers, agronomists, and weed scientists dealing with preventive and curative management of *C. esculentus* across the globe.

2. Materials and Methods

2.1. Genetic Clustering of Belgian C. esculentus Clonal Populations Using the Amplified Fragment Length Polymorphism Analysis (AFLP)

To assess the relatedness and genetic clustering of 35 Belgian *C. esculentus* clonal populations, an AFLP analysis was carried out. In the summer of 2017, mother tubers were collected from 35 different heavily infested conventional maize fields in Belgium (*C. esculentus* coverage of >20%). To include sufficient spatial variation, the selected fields were evenly distributed across the entire area of infestation (30,689 km^2) covering all five Flemish (East Flanders, West Flanders, Antwerp, Limburg, and Flemish-Brabant) and two Walloon provinces (Hainaut and Liège) as shown in Figure 1. The clonal populations were named after the village where they were found; the GPS coordinates and pedohydrological characteristics of the sampling locations can be found in Table 1. The sampling locations were located at least 3 km apart, except Desselgem and Waregem 2 and Herselt 1 and 2, which were located 1 km apart and covered a great diversity of farm and soil types, and hydrological and environmental conditions. Within each infested field, four *C. esculentus* patches (>10 m^2), spaced at least 20 m apart, were sampled by collecting 25 mature daughter tubers from each patch. After merging the samples of each field, 10 tubers of equal size were selected, 6 for genetic analysis and 4 for morphological analysis. From each clonal population, six tubers were planted in pots (9 cm diam.) with one tuber per pot. Within each set of six pots, one pot was randomly selected for genetic analysis. The plants were grown under outdoor conditions from 10 March 2018 until leaf tissue sampling on 16 April 2018 (avg. T_{max} and T_{min} of 12.1 and 4.2 °C, respectively). For each clonal population, 100 mg of fresh green leaf tissue was clipped and put in translucid bags (6.5 × 11 cm). Prior to leaf tissue sampling, all used instruments (forceps, scissors) were pre-rinsed three times with ethanol (95%) to avoid DNA cross-contamination. After leaf sample collection, the bags were immediately frozen in liquid nitrogen and stored in vacuum sealed plastic bags at room temperature in complete darkness.

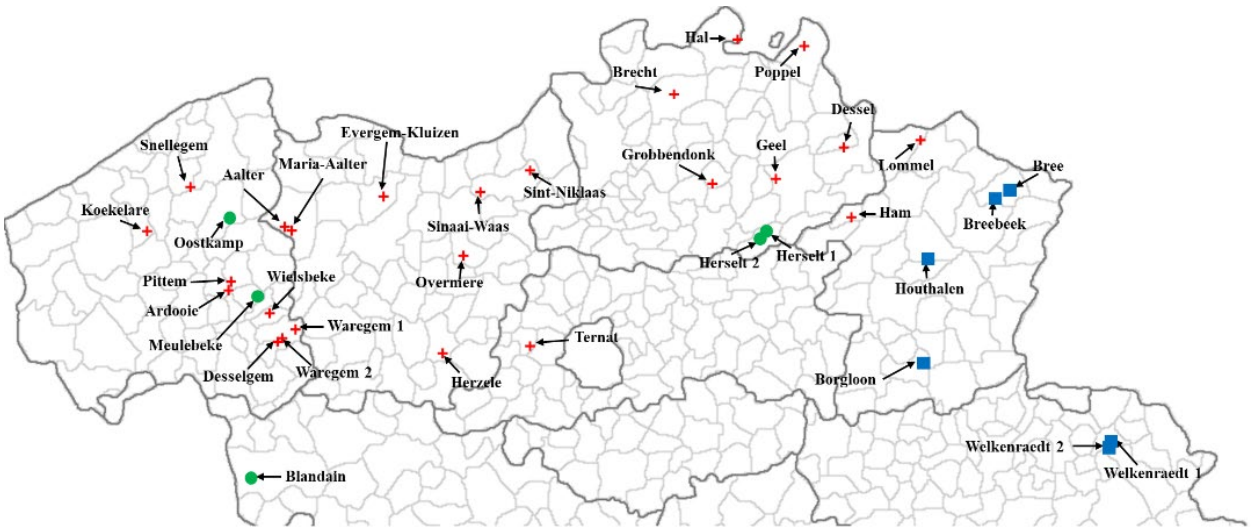


Figure 1. Sampling locations of the 35 *C. esculentus* clonal populations used in the genetic and morphological clustering experiments. Locations are marked with a symbol and colour according to their genetic cluster (red ✚ for cluster A, green ○ for cluster B, and blue □ for cluster C; see Figure 3). See Table 1 for GPS-coordinates and pedohydrological conditions.

Table 1. Sampling locations (township and province) of 35 Belgian *C. esculentus* clonal populations with geographic coordinates, soil texture, and drainage class, derived from the digital soil map of Belgium [25,26].

Origin	Province	Latitude	Longitude	Soil Texture	Drainage Class *
Aalter	East Flanders	51°04′55.90″ N	3°24′25.10″ E	Loamy sand	d
Ardooie	West Flanders	50°58′25.21″ N	3°13′53.39″ E	Sandy loam	d
Blandain	Hainaut	50°37′53.60″ N	3°18′34.80″ E	Loam	b
Borgloon	Limburg	50°49′33.20″ N	5°23′47.30″ E	Loam	b
Brecht	Antwerp	51°21′30.50″ N	4°38′48.20″ E	Loamy sand	d
Bree	Limburg	51°10′02.70″ N	5°38′20.20″ E	Sand	e
Breebeek	Limburg	51°09′26.80″ N	5°36′29.50″ E	Loamy sand	d
Dessel	Antwerp	51°14′38.30″ N	5°07′48.40″ E	Sand	d
Desselgem	West Flanders	50°51′56.94″ N	3°23′22.99″ E	Loamy sand	d
Evergem-Kluizen	East Flanders	51°09′42.30″ N	3°42′49.50″ E	Loamy sand	d
Geel	Antwerp	51°10′45.50″ N	4°55′30.80″ E	Sand	c
Grobbendonk	Antwerp	51°09′19.00″ N	4°44′04.70″ E	Loamy sand	c
Hal	Antwerp	51°26′06.50″ N	4°46′45.90″ E	Sand	c
Ham	Limburg	51°06′08.70″ N	5°10′17.00″ E	Sand	c
Herselt 1	Antwerp	51°04′26.20″ N	4°53′37.00″ E	Sand	c
Herselt 2	Antwerp	51°03′59.20″ N	4°53′28.60″ E	Loamy sand	d
Herzele	East Flanders	50°49′45.80″ N	3°54′49.50″ E	Loam	b
Houthalen	Limburg	51°02′03.30″ N	5°23′47.70″ E	Sand	c
Koekelare	West Flanders	51°04′34.50″ N	2°59′46.87″ E	Loamy sand	d
Lommel	Limburg	51°15′21.20″ N	5°24′10.20″ E	Sand	c
Maria-Aalter	East Flanders	51°04′54.60″ N	3°26′26.90″ E	Sand	d
Meulebeke	West Flanders	50°58′09.70″ N	3°19′13.44″ E	Sandy loam	d
Oostkamp	West Flanders	51°06′46.92″ N	3°14′41.38″ E	Sand	c
Overmere	East Flanders	51°02′08.80″ N	3°56′38.60″ E	Sand	c
Pittem	West Flanders	51°00′00.80″ N	3°13′24.60″ E	Sandy loam	d
Poppel	Antwerp	51°26′24.20″ N	5°01′18.70″ E	Sand	d
Sinaai-Waas	East Flanders	51°09′06.10″ N	4°00′10.20″ E	Sand	b
Sint-Niklaas	East Flanders	51°12′07.90″ N	4°11′04.40″ E	Loamy sand	b
Snellegem	West Flanders	51°09′49.93″ N	3°07′41.31″ E	Sand	b
Ternat	Flemish-Brabant	50°51′22.60″ N	4°10′59.50″ E	Silt loam	a
Waregem 1	West Flanders	50°54′08.96″ N	3°26′59.83″ E	Loamy sand	d
Waregem 2	West Flanders	50°52′14.50″ N	3°22′51.70″ E	Sandy loam	d
Welkenraedt 1	Liège	50°39′49.10″ N	5°55′53.40″ E	Loam	d
Welkenraedt 2	Liège	50°39′20.60″ N	5°56′18.00″ E	Loam	d
Wielsbeke	West Flanders	50°55′40.48″ N	3°21′42.25″ E	Sandy loam	c

* Drainage classes: a, excessively drained; b, well drained; c, moderately well drained; d, imperfectly drained; e, moderately poorly drained.

The DNA was extracted from the plant material following an adjusted NucleoSpin® Plant II protocol from Machery-Nagel [27]. The adjusted quantities and steps are given below. The other quantities and steps were carried out following the manufacturer's recommendations. For cell lysis, step 2b was chosen. In step 2b, 450 µL of Buffer PL2 and 15 µL RNase A was used and the incubation was extended to 30 min at 65 °C. Next, 112 µL of buffer PL3 was used. In step 3, the lysate was first centrifuged before placing the NucleoSpin® Filter. In step 4, 675 µL of PC Buffer was used. In step 5, 600 µL of sample was loaded. The extracted DNA was stored in the freezer at −20 °C. The DNA concentration was measured using a nanodrop spectrophotometer, diluted to a concentration of 15 ng μL^{-1}, and stored at −20 °C until further analysis.

Because of the generation of a large number of reproducible fragments per reaction and in different genomic regions, amplified fragment length polymorphism (AFLP) was chosen to analyse the genome without prior knowledge [28]. These DNA fragments (80–500 bp) can be analysed and result in different banding profiles that can be used to identify genetic variations. AFLP was performed as in De Riek et al. [29] using the commercially available kit from Perkin-Elmer Biosystems (P/N 402083) for fluorescent

fragment detection [28–30]. The primer combinations used were: *Eco*RI-AC/*Mse*I-CAC, *Eco*RI-AC/*Mse*I-CAG, and *Eco*RI-AC/*Mse*I-CAT. The PCRs were run on a GeneAmp PCR system 9700 (Applied Biosystems) and the samples were loaded on an ABI3730*xl* sequencer by Certagen GmbH. The initial band scoring was performed in Genemapper (Applied Biosystems). The data were manually coded for the presence (1) or absence (0) of each band in each plant tested.

2.2. Morphological Clustering of Belgian C. esculentus Clones

To investigate the morphological variation among Belgian clonal populations of *C. esculentus*, an outdoor morphology experiment was conducted. The experiment was a completely randomised block experiment with 35 clonal populations of *C. esculentus* and 4 blocks. To cover sufficient temporal variation, the experiment was repeated during three consecutive years (2018, 2019, and 2020).

The experimental unit was a 9 L round plastic pot filled with a 1:1 mixture of steamed sandy loam and peat. The sandy loam contained 2.6% organic matter, 46.7% silt (2–50 µm), 43.4% sand (>50 µm), and 10.0% clay with a pH-KCl of 5.5. At the start of each growing season, on the 26th of April, a single mother tuber was placed in each pot at a depth of 4 cm. These tubers were pre-sprouted (BBCH 07, i.e., the beginning of sprouting) on a moistened 9 cm diameter filter paper (Rotilabo-Rundfilter type112A, Carl ROTH GmbH, Germany) placed on a germination table ('Arec-cooling technology', Belgium) under a 24/18 °C day/night temperature regime and 16/8 h day/night light cycle. Mother tubers used to establish each clonal population came from the bulked tuber samples from the field (year 2018) or from mature daughter tubers (one from each pot) produced in previous experimental year (years 2019 and 2020). Within a clonal population, the size of mother tubers was kept constant (median size was chosen) to avoid possible maternal tuber size effects on *C. esculentus* growth. Immediately after planting, the pots were placed outdoors on a concrete floor and were irrigated by natural rainfall and overhead sprinklers as needed. Pots were fertilized five times with a two-week interval starting from the 22nd of June. The fertilizer dose was 200 mL of a 1% solution of DCM house and garden (NPK 3-2-5) per pot. Figure 2 shows the daily global radiation and min. and max. daily temperatures for each year, measured by the nearby meteorological station.

At the end of each growing season, on the 24th of September (152 days after planting), the following plant phenotypic/morphological characteristics were determined for each pot: shoot, tuber, and inflorescence number, fresh tuber biomass, individual fresh tuber weight, and number of germinable seeds. The shoot number is the number of shoots with a minimum length of 2 cm. To determine total seed number, inflorescences were clipped 2 cm underneath the lowest bract, air dried, and threshed to collect the mature seeds. After seed cleaning, seeds were counted using a seed counter (Contador seed counter, Pfeuffer GmbH, Germany). After two months of cold storage at 5 °C, the seeds were subjected to a germination test. Per clone, four seed lots of 50 seeds each were exposed to a 25/15 °C day/night temperature and 16/8 h light/darkness regime for 35 days on a germination table. The number of viable seeds was calculated by multiplying total seed number per pot and seed germination percentage and dividing it by 100. After clipping aboveground biomass, all newly formed tubers were washed out of the pot substrate by use of a 200 µm sieve, cleaned, weighted together, and counted. The individual fresh tuber weight was calculated as the fresh tuber biomass divided by the tuber number.

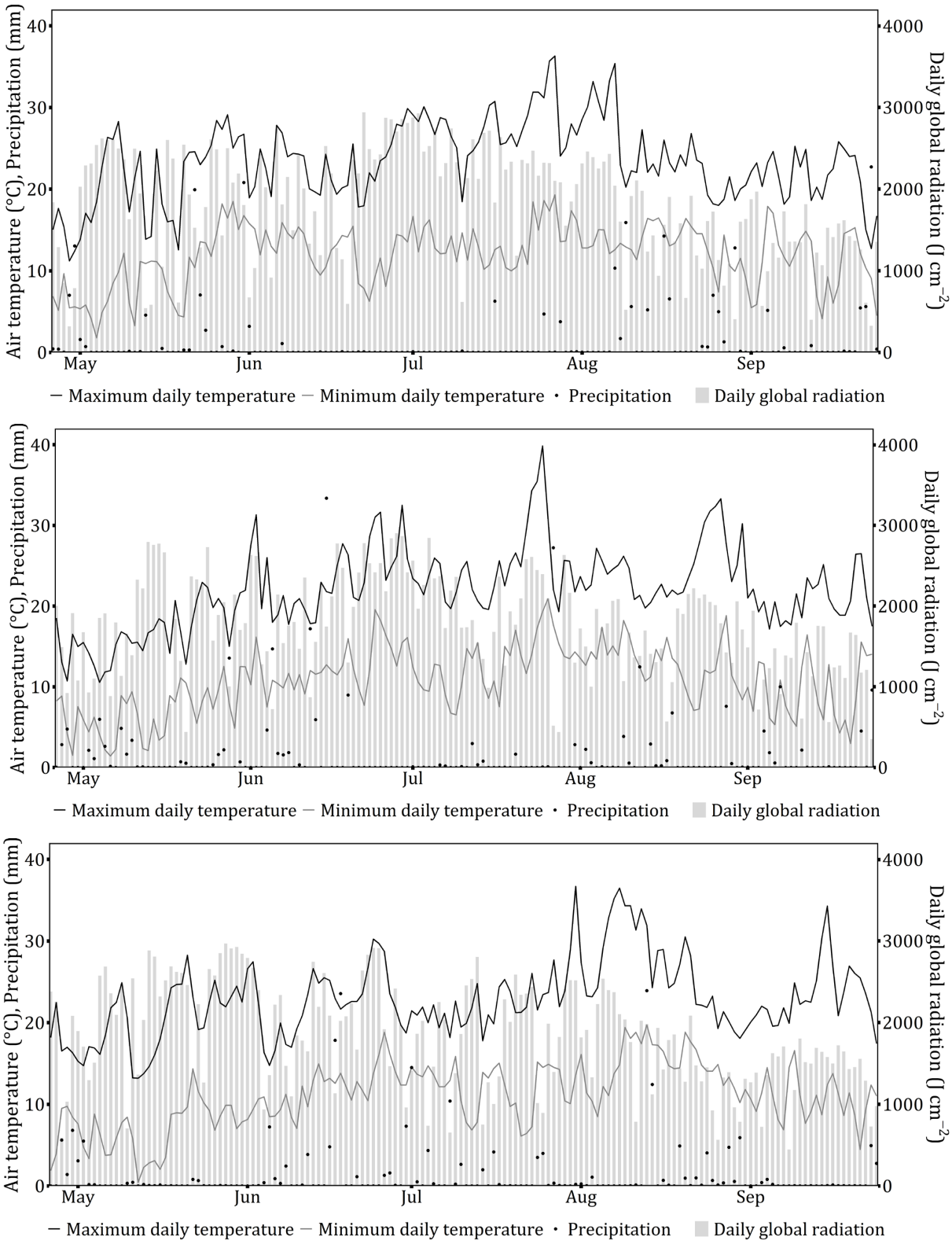


Figure 2. Maximum and minimum daily temperature (°C), precipitation (mm), and daily global radiation (J cm^{-2}) during the experimental period of the morphological experiment in 2018 (**top**), 2019 (**middle**), and 2020 (**bottom**).

2.3. Data and Statistical Analysis

The coded AFLP data were analysed for each primer pair. A scoring table of polymorphic markers was generated for hierarchical clustering of the AFLP data. Monomorphic markers were excluded from the analysis. The polymorphic information content (PIC) was calculated according to De Riek et al. [31]: $PIC = 1 - [f^2 + (1 - f)^2]$ where f is the frequency of the marker in the data set. AFLP-SURV 1.0 [32] was used to calculate the descriptive statistic total gene diversity (H_T). In our study, only one plant was analysed per sampled field as Horak and Holt [33] found that most *C. esculentus* populations were clonal monomorphic populations. Thus, there is no data on the genetic diversity within an entire population of an agricultural field. The Mantel test between Nei's gene diversity and the geographical distance was carried out in R, version 4.0.3 [34]. In SPSS statistics 27 (SPSS Inc., Chicago, IL, USA), the Jaccard index was used for measuring the dissimilarity and the clustering was based on Ward's method [35]. This method was used to create the dendrogram. In addition, in SPSS, a principal component analysis (PCA) was carried out on the genetic data to analyse the variation among the populations.

The morphology data were analysed in SPSS Statistics 27. The experimental factors were the *C. esculentus* clone (35 clones) and year (2018, 2019, and 2020). The normality and homoscedasticity were checked with a Q–Q plot and a Levene test. For each clonal population, the mean (of four replicates) was calculated for each variable and further used in the grouping. A two-factor ANOVA was carried out with the year and clonal population as factors. The grouping of the populations based on the morphological data was done using the hierarchical cluster analysis based on Ward's method. Ward's method was used to minimise information loss associated with grouping. The variables were standardised and a dendrogram was created. For each of the clusters the cluster means were calculated. A one-way ANOVA per variable was used to test for differences. Significance was determined with the Tukey HSD test. Categorical principal component analysis (CATPCA) was performed on the morphology data and generated the CATPCA biplot, component loadings, and correlations between the variables. The CATPCA was used for data reduction and to identify the underlying components of the set of variables measured. The biplot was used to visualize the importance of the contributions of the variables to the principal components and to indicate the correlation between variables. To study the correlations between morphological variables, the Pearson product-moment correlation coefficient was determined.

3. Results

3.1. Genetic Clustering

The AFLP analysis of 35 *C. esculentus* individuals revealed 271 markers, based on three primer pairs (*Eco*RI-AC/*Mse*I-CAC, *Eco*RI-AC/*Mse*I-CAG, *Eco*RI-AC/*Mse*I-CAT). Of these, 207 were polymorphic. This gives an overall polymorphism rate of 76%. The number of markers, polymorphic markers, % of polymorphic markers, and polymorphic information content are given in Table 2. The three primer pairs varied in their degree of polymorphism (70–83%). The total gene diversity (H_T) over the 35 clonal populations was 0.331. The mantel test between Nei's genetic distance and the geographical distance revealed a value of 0.341 with a p-value < 0.05 and 9999 permutations. Hence, the genetic distance between clonal populations was positively and significantly correlated with their geographical distance.

Table 2. Number of markers and polymorphic markers, percentage of polymorphic markers, and polymorphic information content (PIC).

Primer Pair	Number of Markers	Number of Polymorphic Markers	% of Polymorphic Markers	Polymorphic Information Content (PIC)
E-AC/M-CAC	105	81	77.1	0.32
E-AC/M-CAG	91	64	70.3	0.33
E-AC/M-CAT	75	62	82.7	0.31
Total	271	207	76.4	

Figure 3 presents the dendrogram based on the Jaccard distance matrix of the AFLP analysis using Ward's linkage. The AFLP-based dendrogram clustered the 35 *C. esculentus* clonal populations into 3 clusters, hereafter referred to as A, B, and C, containing 24, 5, and 6 clonal populations, respectively. The clonal populations from cluster C were all located in the provinces of Limburg and Liège while the clonal populations from the other clusters were spread out over Flanders. The clonal populations Waregem 1 and 2, Herselt 1 and 2, and Welkenraedt 1 and 2 were clustered in the clusters A, B, and C, respectively.

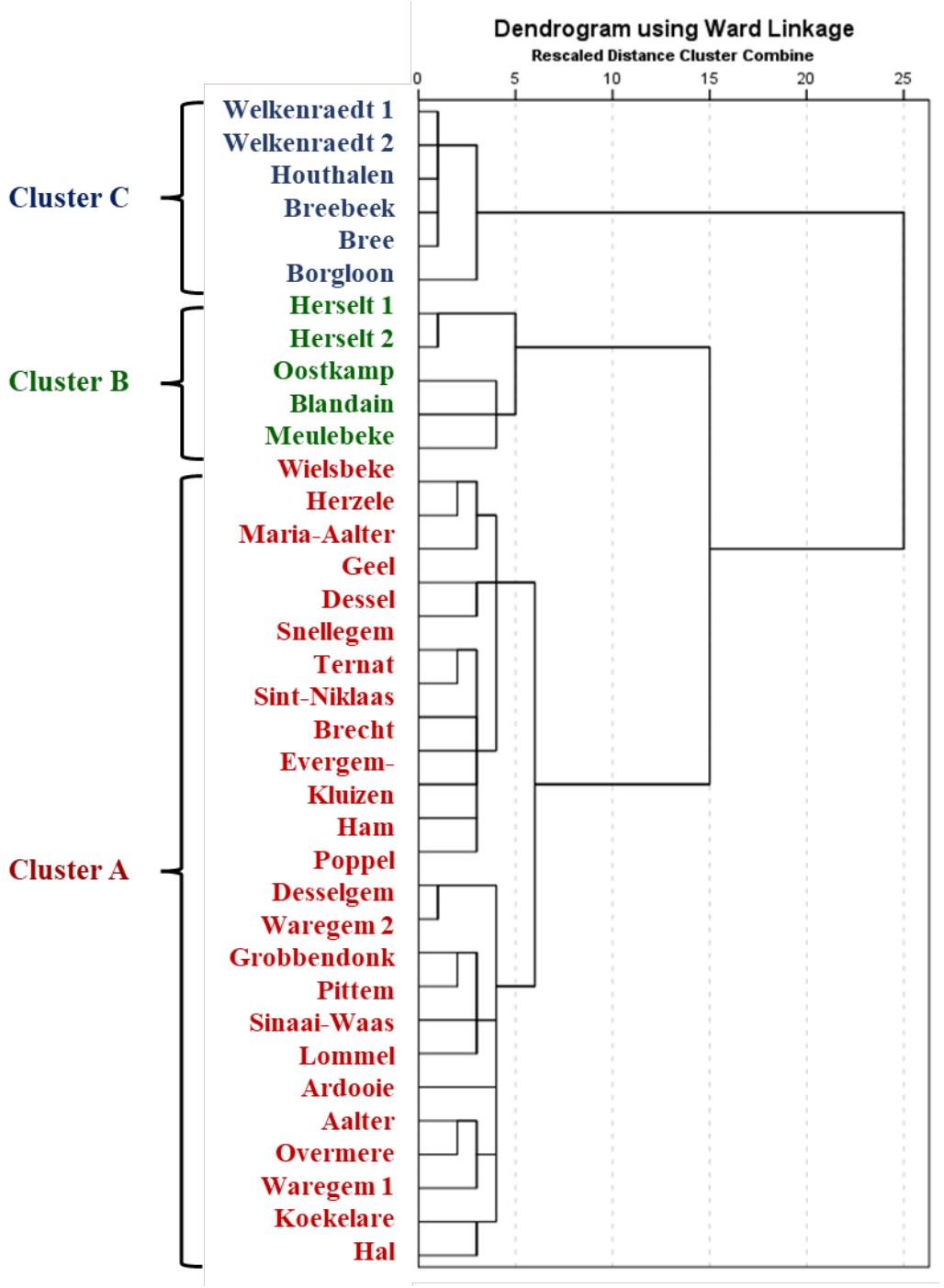


Figure 3. Dendrogram of 35 *C. esculentus* clonal populations clustered by Ward's method based on the Jaccard distance matrix. Three clusters were retained (A, B, and C) based on genetic AFLP analysis.

The PCA analysis (Figure 4) showed three clusters. The first axis (47% of the total variance) discriminated cluster A from clusters B and C. The second axis (17% of the total

variance) discriminated cluster B from cluster C. The third component described 4.4% of the total variance and gave no further separation in or between the clusters. The three clusters thus obtained have the same clonal composition as the genetic clusters provided in the dendrogram (Figure 3).

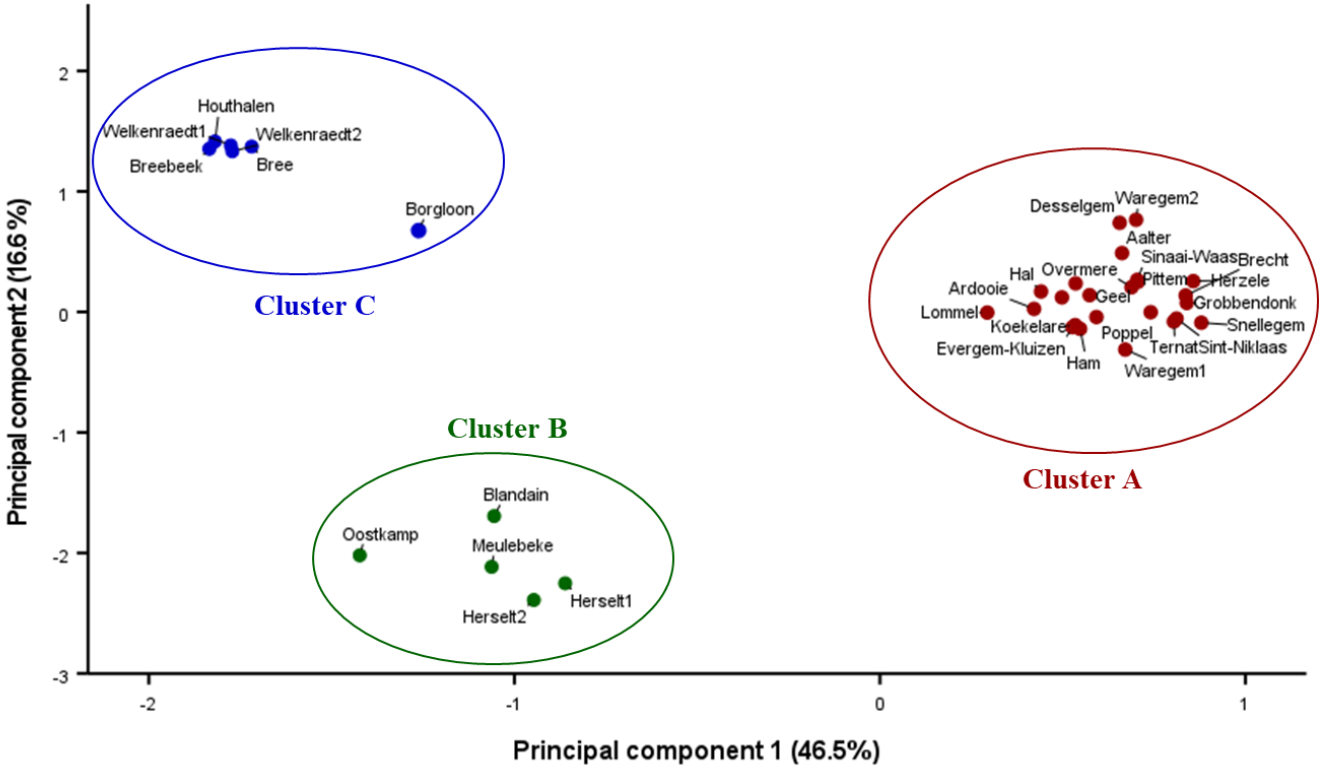


Figure 4. Graphical representation of the 35 *C. esculentus* clonal populations along the first and second principal component axis of the principal component analysis based on the genetic data from the AFLP analysis. The three genetic clusters A, B, and C are marked in red, green, and blue, respectively.

3.2. Morphological Clustering

Based on the morphological data (tuber, shoot, and inflorescence number, fresh tuber weight, individual tuber weight, and number of germinable seeds) a grouping was conducted for each experimental year. Morphological cluster analyses divided the 35 clonal populations into 4 (2018) and 5 clusters (2019 and 2020). Ward's method was used when generating the dendrograms to minimise the information loss associated with grouping. Figure 5 presents the dendrograms. The three genetic clusters (A, B, and C; see Figure 3) are included in the population name in the dendrograms for easy comparison of clusters derived from the morphological and the genetic data. The clustering of the morphological data from 2018 resulted in 4 clusters (identified by numbers 1 to 4), with 11, 11, 5, and 8 clonal populations, respectively. The six clonal populations from cluster C were grouped together in the 2nd cluster while four of the five clonal populations from cluster B were grouped in the 3rd cluster. The majority of the clonal populations (19 out of 24) from cluster A were grouped in clusters 1 and 4. In 2019, the 5 clusters included 10, 1, 11, 6, and 7 clonal populations. The six clonal populations from cluster C were grouped in cluster 3 while four of the five clonal populations from cluster B were grouped in cluster 4 and one in cluster 2. The clonal populations from cluster A were divided over the clusters 1, 3, 4, and 5. In 2020, 5 clusters were retained with 3, 10, 7, 8, and 7 clonal populations, respectively. Again, the six clonal populations from cluster C were grouped together, in cluster 4. All five clonal populations from cluster B were grouped in cluster 3 while the remaining clonal populations from cluster A were divided over the five clusters.

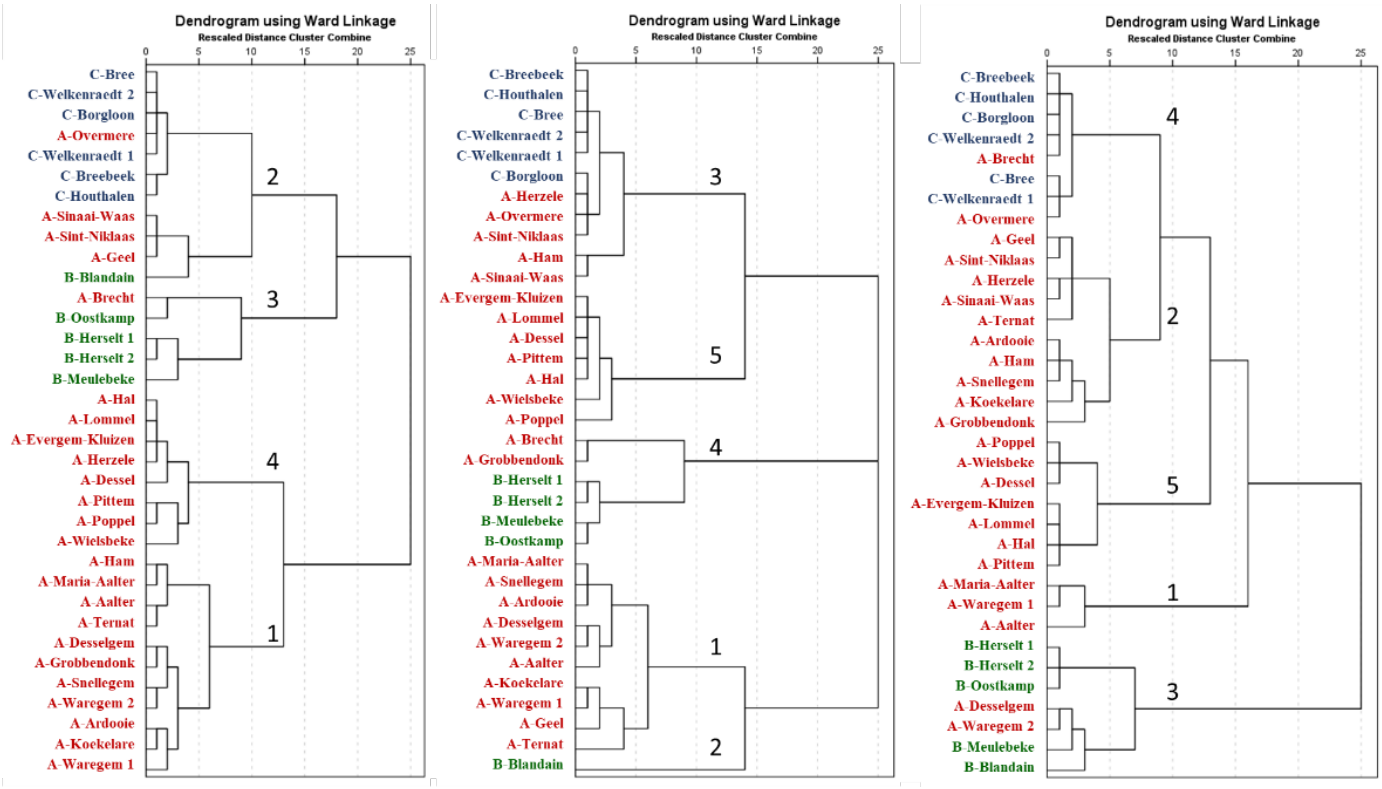


Figure 5. Three dendrograms (one for each experimental year, from left to right: 2018, 2019, and 2020) of 35 *C. esculentus* clonal populations clustered by Ward's method based on morphological traits. Clonal population origin is preceded by a capital letter indicating to which genetic cluster (A, B, or C, see Figure 3) the clonal population is assigned to. Four morphological clusters were retained for 2018 and five for 2019 and 2020.

For each experimental year, a CATPCA biplot was made to illustrate the differences between the clusters. These are given in Figure 6. The three biplots depict the clonal populations, numbered according to the cluster numbers given in Figure 5. Along the first axis of the biplots, the tuber and shoot number on one side and the individual tuber fresh weight on the other side are able to separate the clusters over the three experimental years as the vectors are pointing almost in the exact opposite directions, separating the clonal populations and clusters from one another. Shoot and tuber number were highly correlated, especially in 2019 and 2020. Along the second axis, clusters were separated by means of the fresh tuber biomass on one side and the inflorescence number and germinable seeds on the other side. The biplots show the high correlation between the inflorescence number and number of germinable seeds.

For 2018, the first two principal components (PCs) explained 44% (PC 1) and 35% (PC 2) of the total variance (Figure 6). For 2019 and 2020, this is respectively 42% and 43% (PC 1) and 33% and 34% (PC 2). The component loadings of the two primary principal components for the three years are given in Table 3 and show the importance of each parameter. In 2018, the highest and lowest component loadings for the 1st axis were the variables tuber number (0.95) and individual fresh tuber weight (−0.62). For the 2nd axis these were inflorescence number (0.84) and fresh tuber biomass (−0.57). For the 1st axis in 2019, this was tuber number (0.95) and individual fresh tuber weight (−0.86), and for the 2nd axis, it was viable seeds (0.93) and fresh tuber biomass (−0.44). In 2020, the variables individual fresh tuber weight (0.85) and tuber number (−0.77), and viable seeds (0.74) and fresh tuber biomass (−0.57), had the highest and lowest component loadings for the 1st and 2nd components, respectively.

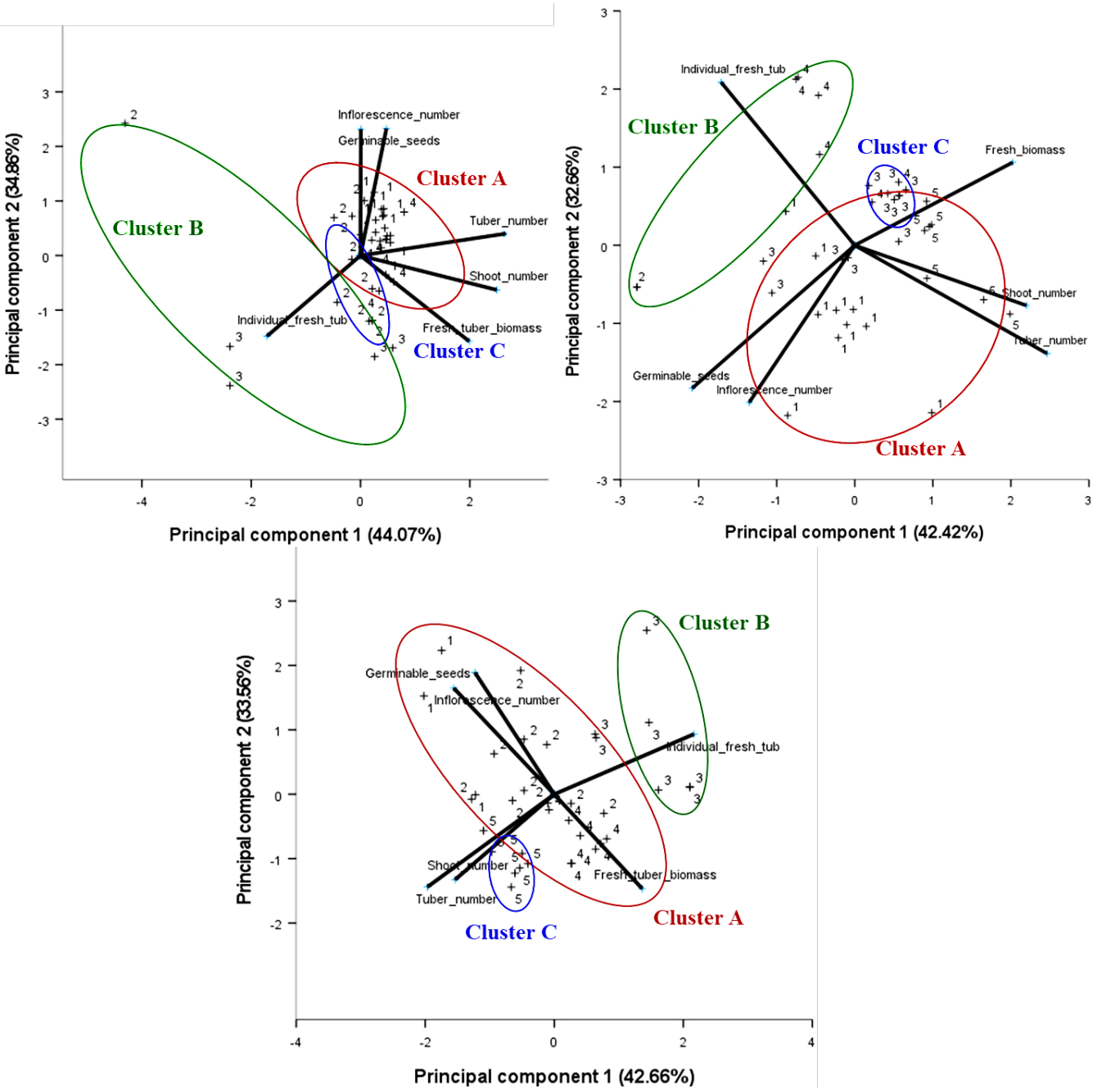


Figure 6. Three biplots (one for each experimental year, top left: 2018, top right: 2019, and bottom: 2020) of 35 *C. esculentus* clonal populations along the first and second principal component axis resulting from categorical principal component analysis on the morphological data. Signs are labelled by morphological cluster (see Figure 5). The three genetic clusters A, B, and C are indicated by red, green, and blue circles, respectively.

Table 3. Component loadings of the first two principal components (PCs) from the categorical principal component analysis (CATPCA) on the morphological variables for the three experimental years.

Variables	Component Loadings					
	2018		2019		2020	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
Tuber number	0.950	0.144	0.948	0.175	−0.769	−0.563
Shoot number	0.901	−0.228	0.797	0.133	−0.601	−0.519
Fresh tuber biomass	0.719	−0.568	0.406	−0.443	0.534	−0.574
Individual fresh tuber weight	−0.618	−0.534	−0.861	−0.350	0.848	0.366
Inflorescence number	0.172	0.842	−0.276	0.851	−0.610	0.645
Number of germinable seeds	0.002	0.838	−0.170	0.932	−0.481	0.740

Table 4 gives the cluster means and standard errors for all morphological variables measured in 2018, 2019, and 2020. Table S1 summarizes the three-year averages (± SE) for each response variable and clonal population. Clusters 4 (2018) and 5 (2019 and 2020) had seven clonal populations in common (Hal, Lommel, Evergem-Kluizen, Dessel, Pittem, Poppel and Wielsbeke) and differed only by the presence of clone Herzele in cluster 4 (2018) (Figure 5). Clonal populations belonging to clusters 4 (2018) and 5 (2019 and 2020) had, on average over their constituting clonal populations, the highest numbers of shoots and tubers but the lowest individual fresh tuber weights, irrespective of experimental year (Figure 5 and Table 4). The clusters (3, 4 and 3 in 2018, 2019 and 2020, respectively) that grouped the clonal populations from cluster B together (Herselt 1 and 2, Oostkamp, Blandain, and Meulebeke) revealed, averaged over their constituting clones, the highest individual fresh tuber weights, irrespective of year (Figure 5 and Table 4). The clonal populations belonging to cluster 1 had each year's highest number of inflorescences. The clonal populations from cluster B, grouped together with cluster 3 in 2018, were characterized by the lowest mean tuber number (419), the highest individual fresh tuber weight and biomass (0.59 and 224.8 g) but also the lowest mean number of inflorescences and number of germinable seeds (2.7 and 219, respectively) (Figure 5 and Table 4). The results were largely confirmed in 2019 and 2020, except for clonal population Blandain that was put in a separate cluster in 2018 and 2019 than the other clonal populations from cluster B. The clonal populations from cluster C, grouped together in cluster 2 in 2018, were characterized by the lowest fresh tuber biomass (150.2 g), a low individual fresh tuber weight (0.27 g), and the lowest number of shoots (42.4) (Figure 5 and Table 4). These results were confirmed in 2019 but were more varied in 2020.

The Pearson's correlation coefficients between the morphological variables for experimental year 2019 are given in Table 5. The Pearson's correlations for the other experimental years (2018 and 2020) were very similar (Tables S2 and S3). Tuber number was strongly positively correlated with shoot number (0.63, $p < 0.01$) but strongly negatively correlated with individual fresh tuber weight (−0.83, $p < 0.01$). The inflorescence number was strongly positively correlated with the number of germinable seeds (0.53, $p < 0.01$). The fresh tuber biomass was negatively correlated with the number of germinable seeds (−0.39, $p < 0.05$) and the shoot number was negatively correlated with the individual fresh tuber weight (−0.40, $p < 0.05$).

Table 4. Cluster code number of *C. esculentus* clonal populations, clonal populations per cluster, mean ± SE for the morphological variables measured per pot, and significance. Cluster means within a year and variable with the same letter are not significantly different at the 5% significance level.

	Cluster	2018			2019			2020		
		Pops./ Cluster	Mean ± SE	Sign.	Pops./ Cluster	Mean ± SE	Sign.	Pops./ Cluster	Mean ± SE	Sign.
Shoot number	1	11	57.9 ± 1.73	b	10	59.1 ± 4.17	b	3	72.7 ± 2.49	b
	2	11	42.4 ± 2.33	a	1	26.0 ± 0.00	a	10	61.1 ± 2.12	ab
	3	5	46.6 ± 5.18	ab	11	45.1 ± 3.03	ab	7	53.0 ± 2.61	a
	4	8	78.8 ± 3.94	c	6	45.6 ± 2.14	ab	8	57.2 ± 1.75	a
	5	-	-		7	90.3 ± 6.46	c	7	86.2 ± 3.67	c
Inflorescence number	1	11	10.9 ± 1.02	b	10	8.9 ± 0.92	b	3	19.7 ± 1.58	c
	2	11	4.0 ± 1.02	a	1	6.0 ± 0.00	b	10	11.4 ± 1.48	b
	3	5	2.7 ± 1.67	a	11	1.4 ± 0.56	a	7	6.4 ± 1.85	ab
	4	8	5.1 ± 1.21	a	6	1.9 ± 0.54	a	8	0.7 ± 0.40	a
	5	-	-		7	1.2 ± 0.25	a	7	5.9 ± 1.32	ab
Tuber number	1	11	641 ± 26.4	bc	10	560 ± 52.4	bc	3	1015 ± 68.2	bc
	2	11	570 ± 52.2	ab	1	221 ± 0.0	a	10	899 ± 59.8	b
	3	5	419 ± 92.4	ab	11	598 ± 28.2	bc	7	501 ± 42.7	a
	4	8	808 ± 62.7	c	6	359 ± 66.5	ab	8	932 ± 30.2	bc
	5	-	-		7	758 ± 53.9	c	7	1165 ± 75.9	c
Fresh tuber biomass (g)	1	11	189.7 ± 7.86	ab	10	128.5 ± 6.19	b	3	155.8 ± 5.26	a
	2	11	150.2 ± 10.38	ab	1	88.1 ± 0.00	a	10	162.1 ± 11.58	a
	3	5	224.8 ± 28.81	b	11	124.6 ± 4.70	b	7	190.7 ± 14.97	a
	4	8	200.6 ± 5.44	b	6	154.2 ± 11.88	b	8	184.2 ± 5.37	a
	5	-	-		7	137.9 ± 3.67	b	7	188.1 ± 10.82	a
Individual fresh tuber weight (g)	1	11	0.31 ± 0.021	a	10	0.25 ± 0.024	a	3	0.15 ± 0.006	a
	2	11	0.27 ± 0.014	a	1	0.40 ± 0.000	b	10	0.19 ± 0.015	a
	3	5	0.59 ± 0.064	b	11	0.21 ± 0.008	a	7	0.39 ± 0.034	b
	4	8	0.26 ± 0.016	a	6	0.47 ± 0.051	b	8	0.20 ± 0.007	a
	5	-	-		7	0.19 ± 0.013	a	7	0.16 ± 0.006	a
Number of germinable seeds	1	11	1043 ± 161.2	b	10	2158 ± 235.5	b	3	6966 ± 2248.4	b
	2	11	431 ± 139.3	a	1	7981 ± 0.0	c	10	1593 ± 398.9	a
	3	5	219 ± 101.9	a	11	566 ± 235.6	a	7	1731 ± 630.0	a
	4	8	479 ± 132.5	ab	6	260 ± 101.4	a	8	21 ± 10.9	a
	5	-	-		7	555 ± 171.1	a	7	612 ± 148.8	a

Table 5. Pearson's correlation coefficients between the morphological variables measured in 2019 on 35 *C. esculentus* clonal populations.

	Shoot Number	Inflorescence Number	Tuber Number	Fresh Tuber Biomass	Individual Fresh Tuber Weight	Number of Germinable Seeds
Shoot number	1.000					
Inflorescence number	−0.010	1.000				
Tuber number	0.626 **	−0.106	1.000			
Fresh tuber biomass	0.235	−0.239	0.163	1.000		
Individual fresh tuber weight	−0.396 *	−0.075	−0.833 **	0.197	1.000	
Number of germinable seeds	−0.171	0.533 **	−0.291	−0.391 *	0.053	1.000

Significance of coefficients is indicated as ** $p < 0.01$, * $p < 0.05$.

4. Discussion

The three primer pairs allowed genetic differentiation of the 35 sampled *C. esculentus* clonal populations of which 31 clonal populations were located in Flanders (13,625 km^2) and 4 clonal populations in Wallonia (16,901 km^2). The total gene diversity (0.33) among the Belgian *C. esculentus* clonal populations (1 plant per clonal population) was higher than the total gene diversity (0.14) among *C. esculentus* field populations (147 plants, 3 plants per field) in a region (Haute Lande, 2448 km^2) in southwest France as found by Dodet et al. [22]. Despite the low gene diversity and the suggestion that only few introductions were involved in Haute Lande, Dodet et al. [22] were still able to genetically differentiate many *C. esculentus* clonal populations. Our genetic analysis revealed three genetically distinct clusters differing in geographical distribution pattern and range. Cluster C exclusively consisted of clonal populations (Welkenraedt 1 and 2, Houthalen, Breebeek, Bree, and Borgloon) from Belgium's easternmost neighbouring provinces (Limburg and Liège). The clonal populations belonging to cluster A were present in all five Flemish provinces (West Flanders, East Flanders, Flemish Brabant, Limburg, and Antwerp). The five clonal populations from cluster B (Herselt 1 and 2, Oostkamp, Blandain, and Meulebeke) are found in three provinces (the neighbouring provinces West Flanders, Hainaut, and Antwerp) and are geographically more separated from one another than the clonal populations from the other clusters. These differential distribution patterns and ranges cannot be attributed to differences in agricultural practices given the similarity in cropping practices and sharing of maize acreage in the total provincial agricultural surface (21–35%) between Flemish provinces [12]. Nor could the clusters be linked to particular pedohydrological conditions such as drainage class and soil texture (Table 1); this is in accordance with Mulligan and Junkins [23] who found *C. esculentus* on different soil textures in North America. Differential geographical distributions of genetic clusters could be linked to their differential morphological profile as discussed further below. The correlation (Mantel test) between Nei's genetic distance and the geographical distance was rather low (0.34), in line with Dodet et al. [22] who found a value of 0.31. Low correlations imply that the genetic differences are probably the result of multiple independent introductions of clonal populations with different genetic backgrounds and/or point to the presence of clonal populations that can easily and quickly spread over large areas, for example, through seeds. In our study, both aspects are deemed important because of the presence of highly distinct clonal populations and the omnipresence of clonal populations belonging to cluster A across Flanders.

The separation of the 35 sampled *C. esculentus* clonal populations into three distinct genetic clusters may indeed point in the direction of multiple (at least three) introductions of *C. esculentus* in Belgium. As about 15,000 arable fields across Flanders are currently infested with *C. esculentus*, the actual total number of genetically distinct *C. esculentus* clusters in Flanders may surmount the number (3) of genetically distinct clusters found in this study based on 35 sampled infested fields, despite being evenly distributed across Flanders and the neighbouring northern part of Wallonia. The high prevalence of genetically distinct clonal populations combined with the ever increasing spread of *C. esculentus* in Belgium (S De Ryck pers. Obs.) increases the probability of finding genetically distinct clonal populations growing in close proximity, which may ultimately increase the probability

of successful sexual reproduction as *C. esculentus* is known as a wind-pollinated, self-incompatible species [36]. Seeds give rise to viable seedlings in situ, in spite of their small size and limited resources [37]. Under these circumstances and provided seeds easily survive winter conditions, sexual reproduction may become the driving force for the formation of new genotypes. Despite their fragile appearance, young seedlings were poorly sensitive to pelargonic acid, bromoxynil, and bentazone when treated 6 weeks after germination and were able to produce tubers that may contribute to further spread [37].

The AFLP analysis resulted in three genetically different clusters and morphological clustering resulted in four to five morphologically distinct groups of clonal populations. The high Jaccard's genetic similarity (>0.62) between populations from the same cluster might indicate that *C. esculentus* is currently largely propagated through asexual means, as a mainly sexual reproduction pattern would most likely lead to higher genetic variability within clusters. However, the Jaccard's genetic similarity had a wider range among clonal populations from clusters A (0.62 to 0.93) and B (0.63 to 0.94) than among clonal populations from cluster C (0.77 to 0.96). The high genetic variability found in clusters A and B, and cluster A in particular, may indicate that sexual reproduction may be substantial as well, in line with Okoli et al. [38] who concluded that the spread over wide areas is mainly done by viable seeds as RAPD analysis (Random Amplified Polymorphic DNA) revealed a high level of genetic heterogeneity in *C. esculentus*. Differences in intra-cluster variability may be caused by differences in fecundity among clusters. Compared to clonal populations from cluster C, clonal populations from cluster A showed up to 204-fold higher numbers of germinable seeds and hence, have higher chance to generate new genetic profiles through the exchange of genetic material with clonal populations from the same or another cluster, provided there is sufficient overlap in flowering period and the clonal populations are in close proximity. This may also explain why clonal populations from cluster A are genetically less related to each other and appear in multiple morphological clusters, together with clonal populations from cluster B or C.

In addition, cluster A also showed a broader spatial geographic spread, as can be seen in Figure 1, than clusters B and C. Owing to its proliferous seed production, cluster A is less dependent on spread via tubers than clonal populations from clusters B and C. Seeds can be carried over longer distances than tubers as they easily survive passage through the digestive tract of fruit and seed-eaters such as waterfowl and terrestrial birds [39]. Given their small size, they attach much more strongly than tubers to machines (e.g., tire crevices, cultivator tines, ploughshares) and shoes. The existence of highly generative clonal populations is of a particular concern for regions with a high share of arable crops that are largely maintained through contracting work, as is the case for Flanders with a high share of maize. When *Cyperaceae* seeds end up in the chopped maize (whole-plant or corn cob mix), they may survive the ensiling process, passage through the ruminant digestive system and manure storage [40], and quickly become dispersed over a wide area by the spreading of the livestock manure.

In contrast with clonal populations from cluster A and B that showed a diffuse geographical distribution pattern, clonal populations from cluster C were strongly geographically delineated (provinces of Limburg and Liège) and genetically quite similar (as indicated by their high Jaccard similarity values between 0.77 and 0.96) indicating that they presumably originated from one single introduction that was locally spread through a number of man-made pathways. A possible reason for the less diffuse spatial distribution pattern of the clonal populations from cluster C relative to the clonal populations from clusters A and B may be their lower fecundity and lower fresh tuber biomass production, the latter being the result of lower individual tuber weight, lower tuber numbers, or their combination. A low fecundity and a low tuber biomass production directly or indirectly restrict the number of viable diaspores (seeds and tubers) that can be dispersed by farm machinery, animals, humans, wind, or water, thus slowing down the rate and extent of spread. Indeed, small tubers generally have small carbohydrate and nutrient reserves and a restricted longevity [41]. The smaller the number of tubers, the smaller the probability that tubers

leave a manifested field with agricultural machinery. The inverse reasoning then leads to the assumption that cluster A is more widespread and diffusely distributed across Flemish provinces relative to clusters B and C. In addition, cluster A had more opportunity to spread itself as it is highly likely that cluster A was the first cluster introduced in Belgium. Indeed, the first documented incidence in 1981 [42] was situated in the area where clone Lommel was sampled. Clonal populations from cluster B had an intermediary position: their dispersion was less diffuse than clonal populations from cluster A due to their lower tuber number and bigger tuber size while being more diffuse than clonal populations from cluster C owing to a higher seed fecundity.

Interestingly, clonal populations sampled from the same municipality were clustered together; they most likely have the same origin and were most likely spread by machinery of local farmers, contractors, or transporters. Follak et al. [43] found that *C. esculentus* is able to move between 3.1 and 5.7 km a year from the centre of the infection and needs vehicles or waterways to move over greater distances. As there is no information on the time that closely related *C. esculentus* clonal populations were first introduced in each of the sampled fields, the initial source of infestation cannot be assigned to a particular field.

The aforementioned low number of genetically distinct clusters and close relatedness of clonal populations from the same municipality suggest that many *C. esculentus* infestations seem to be the result of an accidental introduction of vegetative plant material. Hence, preventive measures such as cleaning machinery, prohibiting the growth of root, tuber, and bulb crops, and cultivating/harvesting/treating infested fields last are still the most effective tactics in combating the spread of *C. esculentus*. However, for clonal populations with high fecundity (cluster A in particular), it is recommended, whenever feasible, to remove all inflorescences before seed set as seeds pose a much higher dispersal risk than tubers.

The clustering based on morphological traits (Figure 5) largely reflected genetic clustering, irrespective of observation year. The vast majority of clonal populations from genetic cluster A (19 in 2018, 17 in 2019, and 20 in 2020 out of 24) clustered in separate morphological clusters that did not contain clonal populations from clusters B or C. This indicates that the morphological traits are largely shaped by genotype and less so by environmental factors or genotype–environment interactions. Nevertheless, some phenotypic plasticity may still occur as some clonal populations belonging to genetic cluster A appeared in morphological clusters grouping clonal populations belonging to genetic cluster B or C. Moreover, the absolute values of the variables did change between the experimental years as seen in Table 4. *Cyperus esculentus* has been known to show great plasticity in morphological variables [18,19]. The differential morphological profiles of genetic clusters may partly explain their differential sensitivity to chemical control methods. De Cauwer et al. [19] showed that the clonal populations with a high individual tuber weight were generally up to 13 and 7.4 times less sensitive to dimethenamid-P and S-metolachlor, respectively, than clonal populations with a lower individual tuber weight. In addition, as discussed above, morphological typing revealed the importance of fecundity, tuber size, and tuber number in explaining particular geographical distribution patterns of *C. esculentus*. The study of De Cauwer et al. [19] was based on a subset of the clonal populations used in our experiments.

5. Conclusions

To conclude, all our research hypotheses were supported, and the AFLP analysis based on 35 Belgian clonal populations sampled across Flanders resulted in three genetically different clusters and four to five morphologically distinct groups of clonal populations. The clustering based on the morphological data (tuber, shoot, and inflorescence number, fresh tuber weight, individual tuber weight, and number of germinable seeds) closely aligned with the genetic clustering based on AFLP data. The morphological typing of the genetic clusters aided in understanding the geographical distribution pattern and rate of spread of particular *C. esculentus* clusters, and in explaining between-field variations in *C. esculentus* control efficacy of herbicide treatments (e.g., dimethenamid-P and S-metolachlor in pre-

emergence, mesotrione + pyridate in post-emergence and glyphosate) reported by farmers and weed scientists. Our results show that it is important for future integrated control systems to take the genetic or morphological differences into account as the invasiveness (e.g., ability to reproduce, rate of spread) and adaptability (e.g., sensitivity to control measures) of *C. esculentus* can vary between, and possibly, within fields as well. For this purpose, morphotyping may be a farmer-friendly, accessible, and affordable method.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020572/s1, Table S1. Three-year averages (± SE) for each response variable and clonal population in the morphology experiment. Assignment of clonal populations to genetic and morphological clusters is indicated by capital letters and digits, respectively. Table S2. Pearson's correlation coefficients between the morphological variables measured in 2018 on 35 *C. esculentus* clonal populations. Table S3. Pearson's correlation coefficients between the morphological variables measured in 2020 on 35 *C. esculentus* clonal populations.

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