

GENETIC ANALYSIS OF *HELOSCIADIUM*  
*REPENS* (Jacq.) W.D.J.KOCH POPULATIONS  
IN GERMANY

—

FUNDAMENTAL RESEARCH FOR  
CONSERVATION MANAGEMENT



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*To my family,*

*...a dwarf standing on the shoulders of a giant may see farther than a giant himself...*



## **PREFACE**

### **Erklärung**

Diese Dissertation wurde im Sinne von §5 der Promotionsordnung von PD Dr. Nikolai Friesen betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungsordnung vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

### **Eidesstattliche Erklärung**

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich / unentgeltlich geholfen.

- SAS Analysendurchführung- JKI Quedlinburg (unentgeltlich)
- Primer design SSR Marker- TraitGenetics GmbH, Gatersleben (entgeltlich)
- Fragmentdetektierung SSR- TraitGenetics GmbH, Gatersleben (entgeltlich)
- Auswahl der Kandidaten für die Analyse bei *H. repens*- in Zusammenarbeit mit Maria Bönisch JKI Quedlinburg (unentgeltlich)
- Auswahl der MAWPs für *H. repens*- in Zusammenarbeit mit Maria Bönisch JKI Quedlinburg (unentgeltlich)
- Materialsammlung wurde durch lokale Sammler durchgeführt (entgeltlich)

Weitere Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Tobias Herden, Osnabrück den

1. Gutachter: PD Dr Nikolai Friesen

2. Gutachter: Prof. Dr Barbara Neuffer



## **Declaration of contribution**

In this thesis, I present the results of my doctoral research, carried out at the Botanical Garden of the Osnabrueck University from March 2015 to April 2019 under the guidance of PD Dr Nikolai Friesen. My thesis resulted in three manuscripts, presented in Chapters 2 to 4. For the paper in Chapters 2 and 3, I compiled the data from the excerpts of the Landesumweltämter (environmental agencies, EA) and data from local botanical institutes. For Chapter 3, I did the lab work (except for the primer design, the fragmentlength detection of the SSRs and the Proc Allele procedure with SAS) and analysed the data. The candidates for the first and second assessment and the the MAWPs were selected in collaborative work with by Maria Bönisch from the Julius Kühn Institute in Quedlinburg. I led the writing of the second manuscript (Chapter 3) with contributions from the co-authors and project-leader Dr Lothar Frese, Julius Kühn Institute in Quedlinburg.

In Chapter 4, I generated all the data and conducted all the analyses. I led the writing with contributions from co-author PD Dr N. Friesen, who also helped to conceive the idea and the theoretical, methodological part.

In Chapter 2, I generated Table 1 and 2 and contributed to the draft. The collection was done by contractors. For Lower Saxony, I acquired most of the contractors, calculated the contracts and organised the permissions from authorities and property owners. Additionally, I managed the first and second assessment of the sites for *H. inundatum* and organised the field collection of material in Lower Saxony. Furthermore, I assessed six sites for *H. inundatum* and one for *H. repens*.

I also wrote an article for the newsletter and one for the webpage of the Botanical Garden of Osnabrueck and gave conference talks and a poster presentation (listed below).

## List of publication

### Peer-view journal articles

**Herden, T.**, Bönisch, M., Friesen, N., 2019. Genetic diversity of *Helosciadium repens* (Jacq.) W.D.J. Koch (Apiaceae) in Germany, a Crop Wild Relative of celery. *Ecology and Evolution*. 00:1-16. DOI: 10.1002/ece3.5947

**Herden, T.**, Friesen, N., 2019. Ecotype or phenotypic plasticity - the aquatic and terrestrial forms of *Helosciadium repens* (Apiaceae). *Ecology and Evolution*. 00: 1-12. DOI: 10.1002/ece3.5833

Frese, L., Bönisch, M., **Herden, T.**, Zander, M., Friesen, N., 2018. In-situ-Erhaltung von Wildselleriearten. *NATURSCHUTZ Landschaftsplanung* 50, 155–163.

### Other publications

Bönisch, M., **Herden, T.**, Nachtigall, M., Friesen, N., Zander, M., Frese, L., 2016: Genetische Erhaltungsgebiete für wildlebende Verwandte der Kulturpflanzen, in: Korn, H., Bockmühl, K., Schliep, R. (Eds.), *Biodiversität und Klima: Vernetzung der Akteure in Deutschland XII ; Dokumentation der 12. Tagung, BfN-Skripten*. Bundesamt für Naturschutz, Bonn-Bad Godesberg, p. 60-62.

**Herden, T.**, 2018: GE-Sell: Das Projekt rund um den Sellerie. Newsletter Freundeskreis Botanischer Garten, Herbst/Winter 2018, 4 S.

**Herden, T.**, 2019: Wussten sie schon? Deutschland hat das erste genetische Erhaltungsgebiet Europas etabliert, online article Webpage of the Botanical Garden of the University of Osnabrueck, URL: [https://www.bogos.uni-osnabrueck.de/Beitrags\\_Vorlagen/2019\\_Wussten\\_Sie\\_schon\\_GE\\_Sell\\_Projekt.html](https://www.bogos.uni-osnabrueck.de/Beitrags_Vorlagen/2019_Wussten_Sie_schon_GE_Sell_Projekt.html)

### Oral presentation

**Herden, T.**, Friesen, N., 2015: Informationsmanagement Kurzüberblick, Symposium und Auftaktveranstaltung “Genetische Erhaltungsgebiete für Wildselleriearten (*Apium* und *Helosciadium*) als Bestandteil eines Netzwerkes genetischer Erhaltungsgebiete in Deutschland“ am 02. Juni 2015, Julius-Kühn-Institut, Quedlinburg.

**Herden, T.**, Westerholt, R., Friesen, N., 2015: Stand der Dinge Universität Osnabrück, Erste Sitzung der projektbegleitenden Arbeitsgruppe (PAG) am 01.12.2015, Bundesanstalt für Landwirtschaft und Ernährung, Bonn.

**Herden, T.,** Mewis, I., Nachtigall, M., Friesen, N., 2016: Markerentwicklung und –auswahl, Zweite Sitzung der projektbegleitenden Arbeitsgruppe (PAG) am 15.12.2016, Bundesanstalt für Landwirtschaft und Ernährung, Bonn.

**Herden, T.,** Westerholt, R., Friesen, N., 2016: Webmapping, Zweite Sitzung der projektbegleitenden Arbeitsgruppe (PAG) am 15.12.2016, Bundesanstalt für Landwirtschaft und Ernährung, Bonn.

**Herden, T.,** Friesen, N., 2018: Stand der Dinge Universität Osnabrück, Dritte Sitzung der projektbegleitenden Arbeitsgruppe (PAG) am 14.03.2018, Bundesanstalt für Landwirtschaft und Ernährung, Bonn.

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**Herden, T.,** 2018: In-situ conservation of species of wild celery – Example for genetic resources of Crop Wild Relatives (CWR). In Schlatti F. (Hrsg.), 2018. 18. Österreichische Botanik-Tagung/24. Internationale Tagung der Sektion Biodiversität und Evolutionsbiologie der Deutschen Botanischen Gesellschaft. – 68. Sonderheft, Naturwissenschaftlichen Vereins für Kärnten, Klagenfurt am Wörthersee, 100 S.

#### **Poster**

**Herden, T.,** Bönisch, M., Friesen, N., 2019: Genetisch Erhaltungsgebiete für *Helosciadium repens* (Jacq.) W.D.J.Koch; Fachtagung Genetische Erhaltungsgebiete für Wildpflanzen für Ernährung und Landwirtschaft – ein neues Modul zur Stärkung des Artenschutzes, 04. and 05. of June 2019, Quedlinburg, Germany, DOI: 10.5073/20190508-131858.



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## Summary

Crop wild relatives (CWR) are an indispensable and at the same time threatened genetic resources for plant breeding. The study uses wild species related to celery to demonstrate how genetic resources of CWRs can be actively maintained in their natural surroundings (in-situ). Genetic reserves should be designated for long term conservation of selected occurrences.

The study presents the selection procedure in detail, aiming at the identification of occurrences and sites suitable for the designation of genetic reserves, the spatial model of a genetic reserve and first practical results of the project. The overall aim of the project is the establishment of a nationwide network of genetic reserves for *Apium graveolens*, *Helosciadium repens*, *H. nodiflorum* and *H. inundatum*, the four wild celery species native to Germany.

*Helosciadium repens* (Jacq.) W.D.J.Koch is threatened by genetic erosion due to a decline in population numbers and sizes. The loss of any population is an irretrievable loss of diversity and opportunity to enhance crops in the future. Genetic reserves are one way to conserve these populations and their genetic potential.

Twenty-seven populations were selected for the analysis in a decision process based on site information. Microsatellites (SSR) were used to elucidate the genetic diversity of German populations. A cluster analysis was performed to see if the individuals form clusters of similarity. For that, a discriminate analysis of principal components (DAPC) was conducted, as the inbreeding index indicated a high number of inbreeding events in the populations and thus discordance with HWE (Hardy-Weinberg equilibrium). The analysis identified six genetic groups, which coincide well with the geographic origin of the analysed plants. The allelic richness (mean counts of alleles per individual per population) was higher in the southern populations compared to the northern ones. This North-South discrepancy was also visible as a high heterogeneity in the cluster assignments in the DAPC analysis. These differences in genetic diversity might be a result of the biogeographic history of Europe, especially the last glacial maximum.

For the establishment of genetic reserves, two populations were considered as most important: The population that differs the most from the average genetic composition and the population that represents the average genetic composition of a population the best. The two extremes of differentiation were interpreted as such that the former has a specific adaptation to its local environment, and the latter represents all populations the best.

DifferInt was used to analyse the SSR data and validate the differentiation of all populations compared to a pool of populations. However, SSRs are not capable of detecting adaptive traits. Populations were additionally chosen from different eco-geographic units (EGU), to increase the chance of capturing different traits. EGUs (Naturräume) are areas of specific abiotic and biotic features. These features may influence selection pressures and induce local adaptations. Based on site parameters and genetic data, 14 most appropriate wild populations (MAWP) were identified for genetic reserves establishment.

For *H. repens*, two eco-forms are known and described in the literature. Besides their different habitats (terrestrial/semi-terrestrial and aquatic) they can be differentiated by morphological traits. Leave and stolon sizes and flowering behaviour differ significantly. Furthermore, the roots of the aquatic forms do not anchor in soil but on other aquatic plants, wood or roots of trees, while the terrestrial form exhibits a shallow root system network similar to other perennial species.

To this end, no genetic analysis was conducted to clarify the phylogenetic status of the putative forms and authors avoided the usage of any specific noun rather than form. The SSR data from the previous study was evaluated, particularly with regards to the two forms. Additionally, an ISSR analysis was conducted, and the data was used to perform a PCA. There was no genetic clustering regarding the two forms neither in the SSR nor in the ISSR data. Nonetheless, the North-South discrepancy in the genetic diversity that was visible in the DAPC plot was confirmed in the PCA of the ISSR data.

However, markers may fail to detect quantitative variation for adaptively important traits. As the most obvious difference in the two habitats was the water availability, the adaptation of both forms to drought stress was studied by measuring the relative water content of leaves, system water content and water loss during drought stress conditions. The stomatal index was measured for different water treatment levels. The results indicate that phenotypic plasticity rather than genotypic adaptation is responsible for different *H. repens* phenotypes.

## **Zusammenfassung**

Wildlebende Verwandte von Kulturarten (WVK) sind eine unverzichtbare und zugleich gefährdete genetische Ressource der Pflanzenzüchtung. Am Beispiel von Wildselleriearten wird dargestellt, wie die genetischen Ressourcen von WVK in ihren natürlichen Lebensräumen (in situ) aktiv erhalten werden können. Zu diesem Zweck werden im Rahmen eines Modell- und Demonstrationsvorhabens Vorkommen identifiziert, die die genetische Diversität im Verbreitungsareal von Wildselleriearten bestmöglich repräsentieren. Für die langfristige Erhaltung dieser Vorkommen sollen genetische Erhaltungsgebiete ausgewiesen werden. Das Verfahren zur Auswahl von Vorkommen und für die Ausweisung genetischer Erhaltungsgebiete geeigneter Flächen, das räumliche Modell des genetischen Erhaltungsgebietes und die ersten praktischen Ergebnisse des Projektes werden im Beitrag dargestellt. Das Ziel des Projektes besteht im Aufbau eines bundesweiten Netzwerkes genetischer Erhaltungsgebiete für Echten Sellerie (*Apium graveolens*), Kriechenden Sellerie (*Helosciadium repens*), Knotenblütigen Sellerie (*H. nodiflorum*) und Flutenden Sellerie (*H. inundatum*), die vier in Deutschland einheimischen Wildselleriearten.

*Helosciadium repens* (Jacq.) W.D.J.Koch ist, aufgrund von Rückgängen in den Populationszahlen und Größen, von genetischer Erosion bedroht. Der Verlust jeglicher Population wäre nicht nur ein unwiederbringlicher Verlust an Biodiversität sondern auch an Möglichkeiten zur Anpassung der Kulturarten für die Zukunft. Genetische Erhaltungsgebiete sind nur ein Weg, um diese Populationen und deren genetisches Potential zu schützen.

Siebenundzwanzig Populationen wurden für die Analyse durch einen gezielten Entscheidungsprozess ausgewählt. Um einen Einblick in die genetische Diversität der Populationen in Deutschland zu bekommen, wurden Mikrosatelliten (SSR) als Marker verwendet. Eine Clusteranalyse wurde durchgeführt um genetische Gruppierungen zu identifizieren. Der  $F_{is}$  Index deutete an, dass es eine hohe Anzahl an Populationen gibt, die von Panmixie abweichen und einen hohen Grad an Inzucht zeigen. Somit weichen sie vom Hardy-Weinberg Gleichgewicht ab. Zu diesem Zweck wurde eine *discriminate analysis of principal components* (DAPC) durchgeführt. Es konnten sechs genetische Gruppen identifiziert werden, die mit der geographischen Verbreitung übereinstimmen. In den südlichen Populationen konnte eine höhere *allelic richness* (durchschnittliche Anzahl der Allele pro Individuum und Population) im Vergleich zu den nördlichen festgestellt werden. Dieser Nord-Süd Unterschied konnte auch in der DAPC Analyse, in Form von Heterogenität der Gruppenzugehörigkeit, in den Populationen im Süden gefunden werden. Diese Unterschiede in der genetischen Diversität der Populationen, kann mit der biogeographischen Geschichte Europas zur Zeit des letzten glazialen Maximums erklärt werden.

Für die Etablierung von genetischen Erhaltungsgebieten sind zwei Populationen von besonderem Interesse. Die Population, die sich am meisten von der durchschnittlichen genetischen Differenzierung aller Populationen unterscheidet, kann als eine an örtliche Bedingungen angepasste Population interpretiert werden. Die Population, die dem Durchschnitt dieser genetischen Differenzierung am besten entspricht, repräsentiert eine, die alle untersuchten Populationen am besten vertritt. Das Programm DifferInt wurde genutzt, um die SSR Daten zu analysieren und die genetische Differenzierung jeder Population im Vergleich zur durchschnittlichen Differenzierung eines Pools von untersuchten Populationen zu bewerten. Um die Möglichkeit verschiedene Anpassungen an lokale Verhältnisse mit einzubeziehen, wurden die Populationen aus verschiedenen Naturräumen ausgewählt. Naturräume sind Gebiete, die aufgrund ihrer biotischen und abiotischen Gegebenheiten zu Räumen zusammengefasst werden. Diese Gegebenheiten beeinflussen den Selektionsdruck und könne lokale Anpassungen induzieren. Basierend auf dem Fundort und den genetischen Daten wurden 14 am besten geeignete wilde Populationen (MAWP) identifiziert und für die Etablierung als genetische Erhaltungsgebiete vorgeschlagen.

Für *H. repens* sind zwei Öko-Formen bekannt, die in der Literatur beschrieben werden. Abgesehen von unterschiedlichen Habitaten (terrestrisch/semi-aquatich und aquatisch), unterscheiden sie sich auch morphologisch. Die Blätter und die Größe der Ausläufer, sowie das Blühverhalten unterscheiden sich sichtlich. Weiterhin wurzeln die aquatischen Populationen nicht im Boden und nutzen ihre Wurzeln vor allem als Anker an anderen Wasserpflanzen, Holz oder Baumwurzeln. Die Terrestrischen wiederum besitzen ein flaches Wurzelsystem im Boden.

Bis heute gibt es keine genetische Analyse um den phylogenetischen Status beider Formen zu klären. Aus diesem Grund vermeiden Autoren taxonomisch wertende Begriffe, und der Begriff „Form“ hat sich etabliert. Die SSR Daten der vorhergehenden Studie wurden bezüglich der beiden

Formen evaluiert. Zusätzlich dazu wurden ISSR Marker eingesetzt und die Daten in einer PCA analysiert. Es konnten keine genetischen Cluster bezüglich der beiden Formen gefunden werden. Die Analysen bestätigen allerdings den Nord-Süd Unterschied, welcher in der DAPC Analyse der SSR Daten gefunden wurde.

Genetische Marker können scheitern beim Detektieren von messbaren Variationen genetischer Anpassung. Der offensichtliche Unterschied beider Habitate ist die Wasserverfügbarkeit. Daher wurde die Anpassung an Trockenstress durch die Messung des relativen Wassergehalts in den Blättern, des System Wassergehalts und des Wasserverlusts während der Trockenstressphasen, ermittelt. Der Stomata Index wurde bei verschiedenen Wasserständen ermittelt. Die Ergebnisse deuten signifikant daraufhin, dass phänotypische Plastizität anstelle von genotypischer Adaption, der Grund für die Ausprägungen der Unterschiede bei beiden Formen ist.

## **Chapter 1:**

### GENERAL INTRODUCTION



## Crop wild relatives

Plant genetic resources (PGR) as defined by the international plant genetic resources institute in Rome, Italy in 1993, are the genetic material of plants that is of value as a resource for the human race now and in the future (IPGRI, 1999). PGR comprises of the cultivated and wild species. Plant genetic resources for food and agriculture (PGRFA) include PGR, which are most directly associated with human food production and agriculture (Maxted et al. 2008). They include resources which contribute to people's livelihoods by providing food, medicine, fodder, fibre, clothing, shelter and energy, to name only a few (FAO, 1997). However, the PGRFA do not include forest plants (Sarah Sensen, BLE (IBV) 2019, personal communication). The PGRFA covers the crops and wild species (EASAC, 2011; FAO, 1997). Therefore, crop wild relatives (CWR) are a subset of the PGRFA with particular value to contribute beneficial traits to crops (Maxted et al. 2006; Maxted and Kell 2009).

The definition of the term CWR is ambiguous due to the continuous refinement during decades of scientific discussions. For instance, the term CWR can be defined as the wild species related to the crops to some degree and crops can be defined as all plants domesticated by humans, such as ornamentals, oil or forage plants (Maxted et al. 2006). However, in most cases the term is used for the wild species related to the crops for the food production only (see BLE (IBV), 2016; Castañeda-Álvarez et al., 2016; Frese et al., 2018; Iriondo et al., 2012; Kole, 2011; Maxted et al., 2008). Between the English- and the German-speaking areas, there are discrepancies in the terminology. Whereas the PGR and PGRFA are the same in both regions, the two terms commonly used in Germany for CWR, WVK (Wildlebende Verwandte der Kulturarten) (Schwand et al. 2009) and WEL (Wildpflanzen für Ernährung und Landwirtschaft) (BLE (IBV), 2016; BMEL, 2015) are not identical in the English translation. While the term WVK is an almost direct translation of the term CWR, the term WEL was formed for political reasons (Sarah Sensen, BLE (IBV) 2019, personal communication) and is commonly used in official sources. Wild species are usually the responsibility of the BMU (Federal Ministry for the environment, nature conservation and nuclear safety). By using the term WEL, the BMEL raised the claim to be responsible for the wild species of the crops (Sarah Sensen, BLE (IBV) 2019, personal communication).

The PGRDEU is a list of PGR for Germany (BLE (IBV), 2016). However, they included forest plants and excluded all crops. The category WEL comprises plants that are considered as CWR but also consists of the wild food plants (WFP – plants that are cultivated as wild species such as *Rubus spec.* or *Allium ursinum*), however, excluding all neophytes (Sarah Sensen, BLE (IBV) 2019, personal communication). Nevertheless, all signatory parties of the FAO agreed that CWRs are essential resources for plant breeding (FAO, 2001). In this study, the term CWR is used for CWR for food and agriculture, and the term WEL is avoided.

## **The values of CWR**

During the domestication, the wild species experienced bottleneck effects, and only a small fraction of the diversity was transferred into the domesticated species (Tanksley and McCouch 1997). This first-generation (in a historical sense) of crops, also called landraces, were still very similar in gene content and resistance against pathogens compared to the wild species. They were adapted to local areas and were sometimes transported into different regions when a certain crop gained importance. Such landraces with secondary centres of origin are often much more diverse than their counterpart in the primary centres (Harlan 1972).

Another bottleneck effect occurred with the introduction of modern crops. They originated from multiple methods such as hybridisation, selection or establishment of inbred lines from the landraces (Harlan 1972; Tanksley and McCouch 1997; van de Wouw et al. 2010). Albeit productive, these modern crops are much more vulnerable to pathogens as the breeding processes narrowed down the gene pool (Harlan 1972; Tanksley and McCouch 1997). By transferring resistance genes or other genetic traits from the wild species into the gene pool of the crops, resistant varieties can be bred (Ochoa and Quiros 1989; Trumble et al. 1990, 1998; Diawara et al. 1992) and the gene pool can be broadened (Veteläinen and Nissilä 2001).

CWRs are those species that are related to the crop (Maxted et al. 2006). Since all plants are related to a recent common ancestor, the question of where to draw the line is justified. According to Maxted et al. (2006), this question was developed in the 1920s by Nikolai Vavilov. In his studies, he namely recognised immense diversity in the Linnean species. In local Russian and Asiatic *Triticum vulgare* Vill., he found 3000 perfectly recognisable varieties (Vavilov 1922). The patterns of variation between crops and their wild relatives in unrelated crop complexes were similar. These patterns were likely to have arisen as a response to similar natural or artificial, or both, selection pressures (Maxted et al. 2006). According to Maxted et al. (2006), Vavilov was one of the first who recognised the importance of conserving the diversity in crops but importantly also within the wild species related to the crops. Harlan and de Wet (1971) formalised the views of Vavilov and developed the gene-pool concept (Maxted et al. 2006).

## Gene pool concept

In this concept, the CWR can be organised into three different groups (Fig. 1) and thus help to determine their value for plant breeding. Among the forms in the primary gene-pool (GP-1) the crossing is easy, and hybrids are fertile. Whereas the crop is listed in the GP-1A, the natural forms are listed in GP-1B. The secondary gene-pool (GP-2) includes all biological species that will cross. Gene transfer is possible but is associated with a struggle to overcome the barriers that separate biological species. The hybrids are mostly sterile. At the level of the tertiary gene pool (GP-3), the barriers cannot be overcome or only with radical methods. The hybrids are lethal, completely sterile or anomalous. Therefore, GP-3 is considered as the outermost border of the CWR (Harlan and de Wet, 1971). However, to assign species to the proposed gene-pool levels, one needs extensive information such as kinship, the pattern of genetic diversity and crossing abilities with the crop (Maxted et al. 2006). In this study, the gene-pool concept was used.

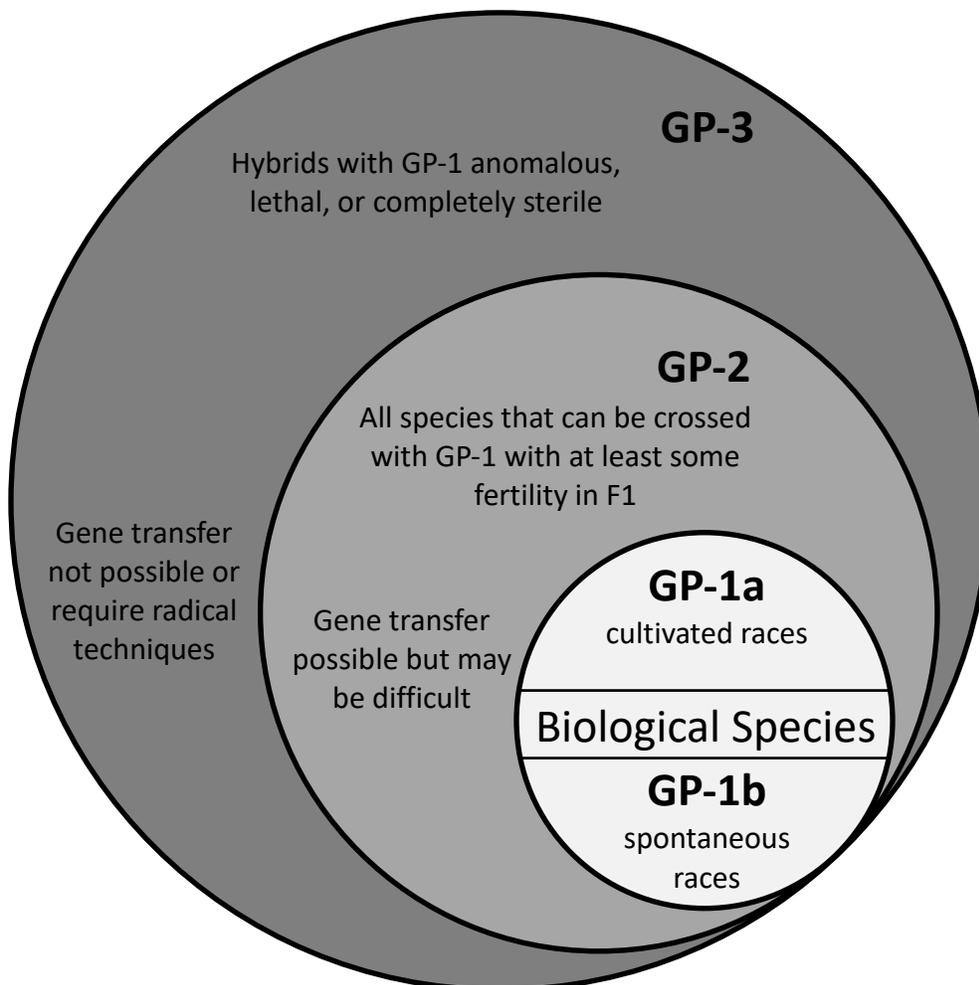


Fig. 1: Schematic diagram of the gene pool concept (recreated after Harlan and de Wet (1971))

## Taxon concept

For CWR with little or no crossing and genetic diversity data available, the taxon concept proposed by Maxted et al. (2006) is appropriate. In such cases, the degree of kinship determines the value of a CWR.

- Taxon Group 1a – crop
- Taxon Group 1b – same species as the crop
- Taxon Group 2 – same series or section as the crop
- Taxon Group 3 – same subgenus as the crop
- Taxon Group 4 – same genus
- Taxon Group 5 – same tribe but different genus than the crop

This concept implies that the taxonomic distance is positively related to the genetic distance, which is not always the case. The taxonomy is, due to different standard genetic distances and methods describing the species, not precisely representing the actual genetic distance but only an approximation of it (Maxted et al. 2006).

## The CWRs of celery and celeriac

The Apiaceae family comprises of more than 3700 species in 434 genera (Stevens 2001). Contrary to their general recognition, the high-level relationships within the family have been challenging to resolve, and many tribes and subtribes within this taxon are not monophyletic (Downie et al. 2001, 2010; Nicolas and Plunkett 2009). The CWR of celery and celeriac are placed within the subfamily Apioideae (Ronse et al. 2010).

There are four CWRs of celery (*Apium graveolens* L. var. *graveolens*) and celeriac (*Apium graveolens* L. var. *rapaceum*) (for simplicity hereafter called celery) listed in the flora of Germany: *A. graveolens* L. ssp. *graveolens*, *Helosciadium inundatum* (L.) W.D.J.Koch, *Helosciadium nodiflorum* (L.) W.D.J.Koch and *Helosciadium repens* (Jacq.) W.D.J.Koch (Jäger 2017) (Fig. 2). Since the revision of Ronse et al. (2010), the CWRs for celery are placed into the two genera *Apium* L. and *Helosciadium* W.D.J.Koch. These two genera are quite distant from one another. The genus *Apium* belongs to the tribe Apieae and is part of the Apiod superclade. *Helosciadium*, on the other hand, belongs to the tribe Oenantheae (Downie et al. 2010). A quick group distance analysis based on ITS sequences with MEGA revealed that the genera *Helosciadium* (*H. repens*, *H. inundatum* and *H. nodiflorum*) and *Apium* (*A. graveolens*) show a between-group mean distance of 0.225 substitutions per site. For comparison, the within-group mean distance in *Helosciadium* is 0.021.

All *Helosciadium* species in Germany are monophyletic (Ronse et al. 2010) and considered to be endangered on different levels (BfN, 2018) (Fig. 2).



Fig. 2: CWRs of celery in Germany at natural sites (modified after Frese et al. (2018)). From left top to bottom right: *A. graveolens*, *H. nodiflorum*, *H. repens*, *H. inundatum*. Red list status (BfN, 2018) is provided at the bottom (RL2- critically endangered, RL3- endangered) (f. l. t. r. and f. t. t. b. U. Meyer-Spethmann (1 and 2), A. Wilhelm, JKI (3), L. Frese, JKI (4)).

Attempts of crossing *A. graveolens* and *H. nodiflorum* were unsuccessful (Pink et al. 1983). As the species *H. inundatum* and *H. repens* are closely related to *H. nodiflorum* (Ronse et al. 2010), one can currently assume a similar crossing result (Frese et al. 2018). The species can be categorised as follows, using the gene-pool concept: GP1- *A. graveolens* (crop and wild form), GP3- *H. inundatum*, *H. nodiflorum* and *H. repens* (Frese et al. 2018).

*Helosciadium repens*, the target taxon in my study, is a small perennial herb which is widely distributed in Western and Southern Europe, parts of North Africa and the Canary Islands (Fig. 3) (Tutin 1968; Hultén and Fries 1986; Ronse et al. 2010; Schoenfelder and Schoenfelder 2012; Muer et al. 2016).

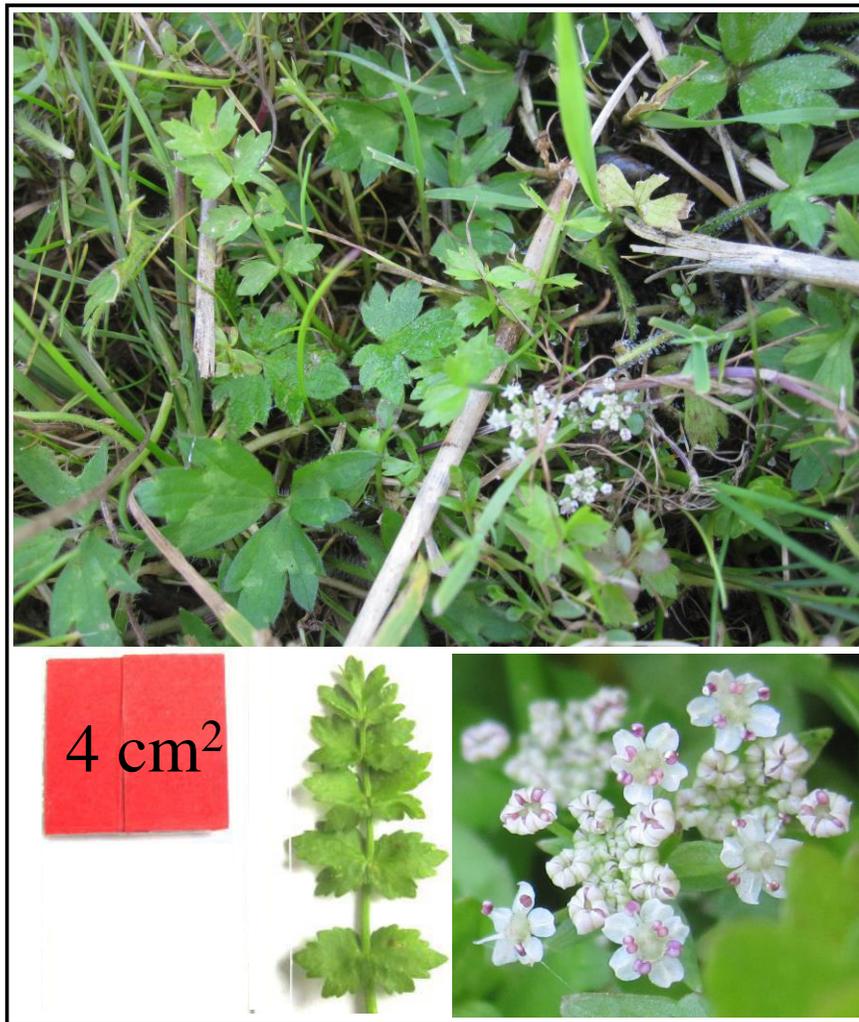


Fig. 3 Habitus of *H. repens* (T. Herden).

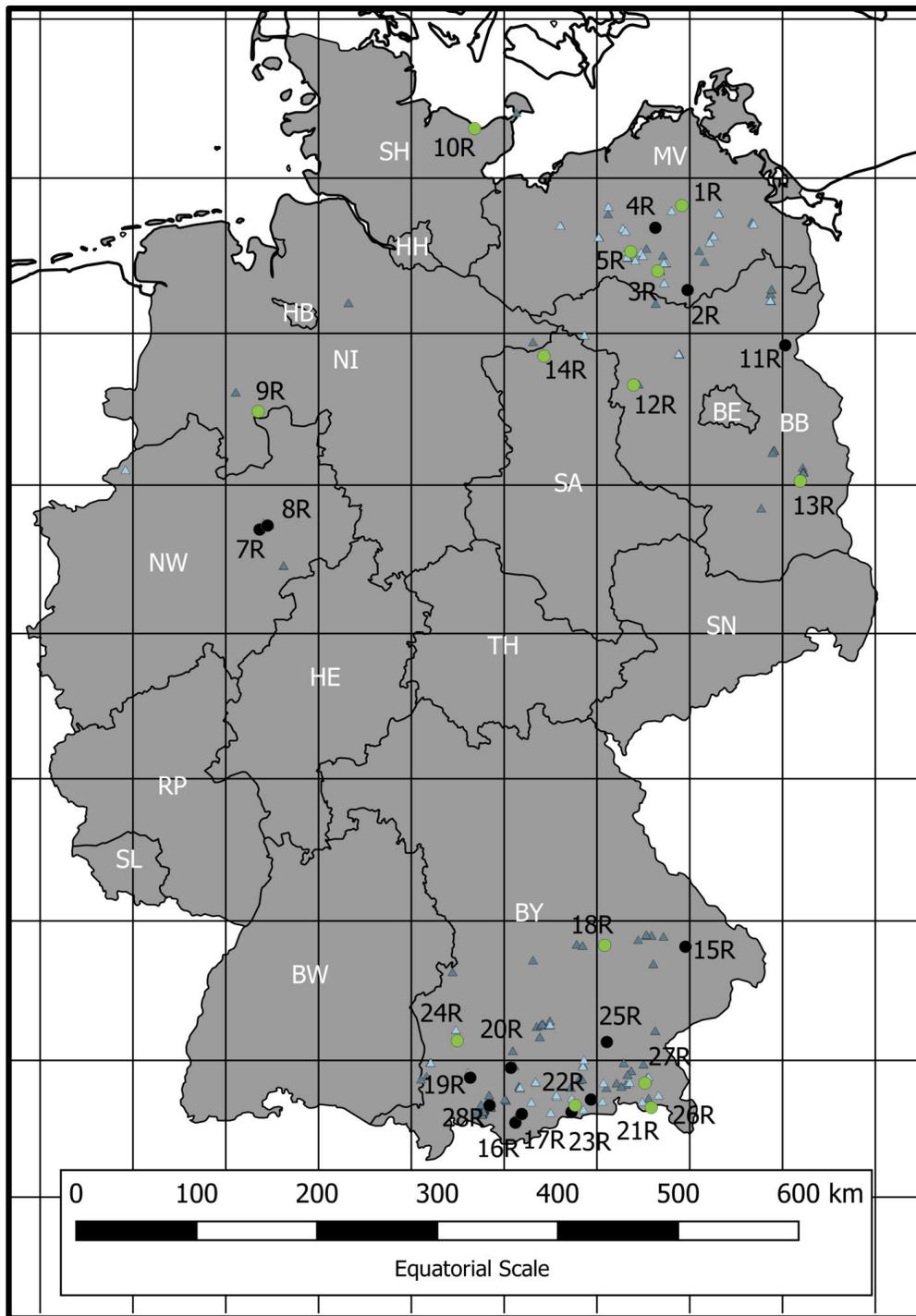


Fig. 4: Provenance of the 27 populations analysed. Dots: analysed populations; green dots: MAWPs; triangles: natural populations in Germany; light blue triangles: preliminary assessed populations in 2015; Federal States of Germany (BB= Brandenburg, BE= Berlin, BW= Baden Wuerttemberg, BY= Bavaria, HB= Bremen, HE= Hesse, HH= Hamburg, MV= Mecklenburg-West Pomerania, NI= Lower Saxony, NW= North-Rhine-Westphalia, RP= Rheinland- Pfalz, SA= Sachsen Anhalt, SH= Schleswig Holstein, SL= Saarland, SN= Saxony, TH= Thuringia); scale bar: at equatorial scale; Pseudo-Mercator-Projection.

In Germany, the distribution area is divided roughly into two parts: the Northern region, with the highest number of populations located in Mecklenburg-West Pomerania (MV), and the Southern region, namely Bavaria (BY) (BfN, 2018b) (Fig. 4).

### **Genetic resources**

There is no doubt that nature should be protected for its own sake, independent of the fact that humans need habitats too. Other reasons such as local recreation and improvement of general living conditions also matter but are rather of public concern. However, the extinction of CWRs represents an irreplaceable loss of genetic resources that might be otherwise beneficial or even vital for crop production and food security (Wehling et al. 2017; Frese et al. 2018). This risk is known to the scientific community since 1920 (Vavilov 1922), but it did not raise as much public awareness as it should have (personal observation).

Since 2017, the world's population reached 7.5 billion and is predicted to reach 10 billion in 2056 (United Nations, 2017). The percentage of arable agricultural land, which is under permanent use, was measured to be around 37% of the global land area in 2016 (The World Bank Group 2019). Since space for agricultural land is limited and competes with conservation, urban and industrial areas, it is of utmost importance to improve farming methods and crops.

However, the means for improvement are at risk. Bilz et al. (2011) evaluated 572 European CWRs and calculated that 11.5% are considered as threatened (vulnerable up to critically endangered) and that for 25% the available genetic data is insufficient. In the later 20<sup>th</sup> century, the signatory parties of the *International Treaty on Plant Genetic Resources for Food and Agriculture* and the *Convention on Biological Diversity* committed themselves to protect CWRs (CBD, 1992; FAO, 2001).

Since then, there has been a considerable improvement in conserving CWRs *in-situ* and *ex-situ*. In 2015, the European cooperative program for plant genetic resources presented a concept for conservation of CWRs (Maxted et al. 2015). The federal ministry for food and agriculture entered the importance of CWRs into the national program of plant genetic resources in Germany (BMEL, 2015). The second target of the so-called Agenda 2030 is to focus on stopping worldwide hunger by creating resilient crops and maintain genetic diversity in food production (BMZ, 2017; Rosa, 2017).

However, more needs to be done. Mounce et al. (2017) estimated that there are over 3,200 seed collections in botanical gardens in 180 countries. However, for 29.1% of the CWRs, no germplasm accession exist, and 23.9% are represented only by fewer than ten accessions (Castañeda-Álvarez et al. 2016).

### **Genetic reserve**

One way to protect CWR is *ex-situ* conservation (FAO 1975; Hurka et al. 2004; Li and Pritchard 2009; Borgmann et al. 2014). The collection and storage of germplasm are manifold, going from

the maintenance of clonal crops in field gene banks and *in-vitro* banks, conservation stands of tree species (Li and Pritchard 2009), to seed-bearing species in botanical gardens and seed banks (Hurka et al. 2004; Borgmann et al. 2014). One of the advantages is that the species are conserved outside and in distance of the threats, they might have faced in their natural habitat (Li and Pritchard 2009). The costs for this method is estimated to be 1% of that of *in-situ* conservation (Li and Pritchard 2009). Nonetheless, facilities (such as chest freezer or walk-in cold stores), space for cultivation and staff is needed (Hurka et al. 2004; Li and Pritchard 2009; Borgmann et al. 2014). The downside is that a seed collection only reflects a state of the genetic diversity of a population or species in space and time.

Much more expensive and time-consuming is *in-situ* conservation (Li and Pritchard 2009). This method can be divided into two techniques. When farmers sustainably manage locally developed traditional crop varieties within traditional agricultural practices, one speaks of on-farm conservation (Maxted et al. 1997). They are exposed to the local environment and can develop traits such as resistance and resilience against pathogens and pests (Borgmann et al. 2014). They are managed in the same surroundings where they have developed their distinctive properties (Maxted et al. 2015).

Contrarily, genetic reserve conservation takes place in natural wild populations within defined areas (genetic reserves) (Maxted et al. 1997). A genetic reserve, as described by Maxted *et al.* (1997), is an area where the genetic diversity of natural populations is monitored and managed for long-term conservation. Sufficient size of the biotope or ecosystem, do not prevent the evolution of the species, and they can develop adaptations through natural selection (Borgmann et al. 2014). However, natural and human-made disasters can still endanger those protected populations (Li and Pritchard 2009). Therefore, it is reasonable to combine both methods of conservation (*ex-situ* and *in-situ*) and store seeds from genetic reserves periodically in seed banks (Maxted et al. 1997).

The purpose of a genetic reserve is to conserve the genetic intra-specific diversity and regional genetic patterns. Multiple, spatial distributed genetic reserves are necessary to get a representative sample of the complete genetic diversity in a defined distribution area. The selection needs to be based on the spatial patterns of genetic diversity (Maxted et al. 1997). Iriondo et al. (2012) proposed quality standards to decide which population is the most appropriate wild population (MAWP) for establishing a genetic reserve. S. Kell defined the term MAWP (Maxted et al. 2015) which described an *in situ* conservation unit selected according to the proposed quality standards for genetic reserves of Iriondo *et al.* (2012).

Hawkes et al. (1997) discussed a theoretical model for a genetic reserve which they adapted from Cox (1993) (modified from Batisse (1986)). This model establishes different overlapping zones with varying goals of management. In the central core area, which should contain a stable habitat for the target species, the management is strict, and no measures are allowed that could harm the target species. The buffer zone surrounds this zone with potential habitats between the core zones where the species could flourish. It should protect the core from edge effects. Within the buffer

zone, the management is equally strict. A socio-buffering would allow sustainable agriculture and forestry and may be advisable if locals lost traditional harvesting right in the core zone. However, Hawkes et al. (1997) stressed that close monitoring of the local activities is needed if socio-buffering is used. The transition zone embeds all zones mentioned above, plus an area where limited human settlements and sustainable utilisation, as well as general tourist visits, are allowed (Hawkes et al. 1997). It represents the outer margin of the genetic reserve.

## Research questions

The primary goal of this study was to find the most appropriate wild populations (MAWP) in Germany as candidates for genetic reserves of the CWR *H. repens* (Chapter 3). Therefore, I analysed the genetic diversity of 27 populations with microsatellites (SSR) and combined the results with the parameters recorded during the assessment of the sites.

Several selection phases were needed to get to the point of analysing (see Chapter 3 for detailed selection criteria). First, I compiled a list of all data excerpts for all four species provided by the Landesumweltämter (environmental agencies, EA) and data from local botanical institutes. In cooperation with Maria Bönisch from the Julius Kühn Institute in Quedlinburg, most of the candidates for the first assessment were selected. Contractors such as local experts or representatives of the local botanical institutes visited the sites. I acquired contractors for Lower Saxony, calculated the proposals and organised the permissions from authorities and property owners, and oversaw the first and second assessment and the collection of material in Lower Saxony. Additionally, I visited six sites for *H. inundatum* and one for *H. repens* (Ochsenmoor, Lower Saxony) by myself and collected material. The contractors were instructed to give a preference ranking of the population sites.

Sites were chosen using the data from the sites visits and the preference ranking. This work was done in cooperation with Maria Bönisch as well as the selection of the MAWPs.

We wanted to know the genetic differentiation of *H. repens* populations in Germany. Furthermore, the analysis gave us an inside in the genetic composition and possible conclusions regarding the reproduction strategy. However, the main reason for the genetic analysis was to infer which population is most differentiated from all others and which one was the “average Joe” and this represented the genetic diversity of all the investigated populations the best. Those two populations would represent the first and most important MAWPs in the selection (Chapter 3). The decision for the rest of the 14 MAWPs was based mainly on feasibility, cost-effectiveness and site parameters as described in Iriondo et al. (2012) (Chapter 3).

For the second part of this study, I focused on the ecology of the species (Chapter 4). In literature, there are two recognised and described forms for *H. repens*. One form that roots in the soil and can be described as terrestrial or semi-aquatic and one that exclusively floats in small streams and rivers and uses their roots to anchor on wood, tree roots or other aquatic plants to stay immobile. Until now, their taxonomical status has not been validated with genetic data. The research question was

if those described differences are caused by variations in the genome or are the results of phenotypic plasticity.

For the genetic differentiation, the SSR data from Chapter 3 was analysed with a focus on the two forms and an additional analysis using ISSRs was carried out. As water availability is the central difference between both habitats, an experiment where the vitality during drought stress and the adaptation between two drought periods were measured, was carried out. Both molecular and morphological data were used to solve the two above mentioned questions.



## **Chapter 2**

IN-SITU-ERHALTUNG VON WILDSELLERIEARTEN.

FRESE, L., BÖNISCH, M., HERDEN, T., ZANDER, M., FRIESEN, N.

NATURSCHUTZ UND LANDSCHAFTSPLANUNG, 2018, **50(5)**:155–163.



### Chapter 3

GENETIC DIVERSITY OF *HELOSCIADIUM REPENS* (JACQ.) W.D.J.  
KOCH (APIACEAE) IN GERMANY, A CROP WILD RELATIVE OF CELERY.

HERDEN, T., BÖNISCH, M. AND FRIESEN, N.

*ECOLOGY AND EVOLUTION*, 2019; 00:1-16.

DOI: 10.1002/ece3.5947



## ORIGINAL RESEARCH

# Genetic diversity of *Helosciadium repens* (Jacq.) W.D.J. Koch (Apiaceae) in Germany, a Crop Wild Relative of celery

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Email: therden@uni-osnabrueck.de**Funding information**

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**Abstract**

- *Helosciadium repens* (Jacq.) W.D.J. Koch is threatened by genetic erosion. It is a Crop Wild Relative (CWR) of celery and celeriac and a potentially valuable genetic resource for plant breeding. The objective of this study was the analysis of distribution of genetic diversity with a set of selected populations in Germany. The results of the genetic analysis and data obtained during the site visits were used to identify a subset which was chosen to best represent the genetic diversity of *H. repens* in Germany. The chance of long-term conservation by securing the identified populations in genetic reserves is distinctly possible.
- Seven hundred and fifteen individuals from 27 sites were assessed using six simple sequence repeat markers. Discriminant analysis of principal components was used to identify six clusters of genetically similar individuals. The complementary compositional genetic differentiation  $\Delta_j$  was calculated to designate a subset of populations chosen to best represent the overall genetic diversity. Entry 18R ( $\Delta_{18R} = 0.2498$ ) represented its pooled remainder the best, while entry 22R ( $\Delta_{22R} = 0.4902$ ) differed the most from its complement.
- Based on the results of the genetic analysis and information regarding the current conservation status, 14 most appropriate wild populations for potential genetic reserve were identified. The used markers display a low level of genetic variation between the analyzed populations, and a split between Northern and Southern populations was observed.
- CWR species are essential genetic resources for plant breeding and food security. However, 11.5% of the European CWRs are threatened. Therefore, it is of utmost importance to determine their genetic compositions. These insights will provide the fundamental basis for making crucial decisions concerning future conservation strategies for *H. repens*.

**KEYWORDS**Crop Wild Relatives, genetic diversity, genetic reserves, *Helosciadium repens*, most appropriate wild population, SSR

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## 1 | INTRODUCTION

Crop Wild Relative (CWR) species are, to some degree, related to the crops we use today. They often contain valuable resistance genes and other useful genetic traits and are thus essential resources for plant breeding (Hajjar & Hodgkin, 2007; Kole, 2011). When crossing the wild species with the crops, more resilient varieties can be bred (e.g., Diawara, Trumble, Quiros, & Millar, 1992; Martín-Sánchez et al., 2003; Ochoa & Quiros, 1989; Paula, Dinato, Vigna, & Fávero, 2019; Simmons, Jarret, Cantrell, & Levi, 2019; Trumble, Derecks, Quiros, & Beier, 1990; Trumble, Diawara, & Quiros, 1998) which would contribute to broadening the breeding pool (Veteläinen & Nissilä, 2001).

The increase in the world's population, which is predicted to reach 10 billion in 2056 (United Nations, 2017), accompanied by a decrease in arable agricultural land (The World Bank Group, 2019) and forecast changes in climate, drives the needs of agriculture to enhance the productivity of crops (Henry, 2014; Shapter et al., 2013). However, finding the means to effect this enhancement is at risk. Of the 572 European CWRs assessed in a study by Bilz, IUCN Regional Office for Europe, and IUCN Species Survival Commission (2011), 11.5% are threatened (vulnerable to critically endangered) and for 29%, the available genetic data was insufficient (Bilz et al., 2011). The loss of these genetic resources will have unpredicted consequences for crop production and food security (Frese, Bönisch, Herden, Bönisch, Herden, Zander, & Friesen, 2018; Henry, 2014; Wehling, Scholz, Ruge-Wehling, Hackauf, & Frese, 2017). There is, therefore, considerable interest in agricultural policies directed at protecting genetic resources in situ and ex situ (BMEL, 2015). Already, in the later 20th century, the signatory parties of the *International Treaty on Plant Genetic Resources for Food and Agriculture* and the *Convention on Biological Diversity* committed themselves to the protection of CWRs (CBD, 1992; FAO, 2001). The model and demonstration project "Genetische Erhaltungsgebiete für Wildsellerie (*Apium* und *Helosciadium*) als Bestandteil eines Bundesweiten Netzwerkes genetischer Erhaltungsgebiete in Deutschland- GE-Sell" (Genetic Nature Reserves for Wild Celery (*Apium* and *Helosciadium*) as Part of a National Network in Germany) is one of the few projects, attempting to establish genetic reserves in practice (Frese, Bönisch, Herden, et al., 2018).

There are two main approaches to categorizing CWR in relation to their crops: The gene pool concept (Harlan & de Wet, 1971) and the taxon concept (Maxted, Ford-Lloyd, Jury, Kell, & Scholten, 2006). The approach of Harlan and de Wet (1971) is based on crossability between the crop and the CWR and was applied in the above-mentioned project (Frese, Bönisch, Herden, et al., 2018). In Germany, four wild celery species are considered to be CWR of *A. graveolens*: *A. graveolens* L. ssp. *graveolens*, *Helosciadium repens* (Jacq.) W.D.J. Koch, *Helosciadium inundatum* (Jacq.) W.D.J. Koch and *Helosciadium nodiflorum* (Jacq.) W.D.J. Koch. Pink et al. (1983) had no success in their attempt to cross *A. graveolens* crops with *H. nodiflorum*. There have as yet been no attempts at crossing the crop with *H. repens*. Since *H. repens* is closely related to *H. nodiflorum* (Ronse, Popper, Preston, & Watson, 2010), Frese, Bönisch, Herden, et al. (2018) advocated a temporary classification into the tertiary gene pool of

*A. graveolens*. This gene pool represents the extreme outer limit of the potential gene pool of the crop (Harlan & de Wet, 1971).

*Helosciadium repens* belongs to the Apiaceae family. It is a small perennial herb which is widely distributed in Western and Southern Europe, parts of North Africa and the Canary Islands (Hultén & Fries, 1986; Muer, Sauerbier, & Cabrera, 2016; Ronse et al., 2010; Schoenfelder & Schoenfelder, 2012; Tutin, 1968). Despite its broad distribution area, the species is scarce and listed as near threatened in Europe (Bilz et al., 2011). It is also considered critically endangered in Germany classified with different levels of endangerment across the federal states (BfN, 2018a, 2018b). In Germany, the distribution area is divided roughly into two parts: The Northern region, which has the highest number of populations located in Mecklenburg-West Pomerania (MV), and the Southern region, namely Bavaria (BY; BfN, 2018a). Even though *H. repens* has never been an abundant species in general (Burmeier & Jensen, 2008), its distribution area began to decline due to urbanization and changes in land-use. This habitat shrinkage will continue in the future if model scenarios prove to be correct (Aguirre-Gutiérrez, Treuren, Hoekstra, & Hintum, 2017; Burmeier & Jensen, 2009). The species is hemicryptophytic (Oberdorfer, 1983; Schubert & Vent, 1994). However, hydrophytic populations with their submerged hibernating organ can be found occasionally (Casper & Krausch, 1981; NLWKN, 2011; Schossau, 2000, cited in Hacker, Voigtländer, & Russow, 2003). It grows on alternating wet pastures, littoral zones of trenches and springs (Weber, 1995) and along slow running streams. Furthermore, populations growing in stagnant water can also be found.

This plant is a weak competitor against taller herbs or shrubs as it is light-demanding and low-growing. As a consequence, *H. repens* can often be found on mowed lawns at camping grounds, or areas with grazing management (Burmeier & Jensen, 2009; McDonalds & Lambrick, 2006). Due to its creeping stolon habitus, it occupies uncovered ground very quickly. However, even slight changes in grazing management which benefit its competitors can lead to drastic changes in population sizes (e.g., a shift of livestock or change in mowing periods). Should this be the case, populations can gradually disappear over several vegetation periods (Burmeier & Jensen, 2008, 2009; Naturschutzring Dümmer E.V., 2015 unpublished data). *Helosciadium repens* propagates not only clonally but also by seeds (Burmeier & Jensen, 2008; Hacker et al., 2003). It produces numerous self-compatible flowers which produce nectar to attract small insects (East, 1940; Frank & Klotz, 1990; Ronse et al., 2010). From these monoicous, facultatively xenogamous flowers, two seeds are produced which have no mechanisms for long-distance dispersal (Klotz, Kühn, & Durka, 2002; Lederbogen, 2000). However, endozoochoric propagation from birds is possible (Lederbogen, 2000). Additionally, the seeds can stay afloat for approximately 24 hr and are thus able to drift for at least short distances (Burmeier & Jensen, 2008). Dormant seeds build seed soil banks from which the species can recruit seedlings once there are gaps in the vegetation cover or less competition (Burmeier & Jensen, 2008).

The primary goal of this study is to find the most appropriate wild populations (MAWP) as candidates for genetic reserves of one of the

CWR of *A. graveolens*: *H. repens*. The term MAWP was defined by S. Kell (Maxted et al., 2015) and describes an in situ conservation unit selected according to the proposed quality standards for genetic reserves of Iriondo et al. (2012).

A genetic reserve, as defined by Maxted, Hawkes, Ford-Lloyd, and Williams (1997), is an area where the genetic diversity of natural populations is monitored and managed for long-term conservation and captures as much of the genetic diversity of the target taxon as possible (Iriondo et al., 2012). For this, we characterized selected populations of *H. repens* in Germany with microsatellites (SSR). To understand the contribution of each population to the overall diversity within the entire set, we analyzed the genetic diversity and composition of 27 occurrences. Finally, MAWPs were chosen, using criteria based on the quality standards proposed by Iriondo et al. (2012). The required habitat, site, population, legal, social, and management data were recorded during the site visits. At the end of an eight-step planning process (Frese, Bönisch, Herden, et al., 2018), we propose to establish genetic reserves for 14 MAWPs.

## 2 | MATERIAL AND METHODS

### 2.1 | Preselection of occurrences

A list of distribution data of *H. repens* in Germany was created with the help of database excerpts provided by the Landesumweltämter (environmental agencies, EA) and data from local botanical institutes. The heterogeneous data set was homogenized in order to make the records comparable. The inventory contained 1,040 entries, of which 78 populations were selected for a preliminary assessment. Populations were selected based on the following criteria. (a) The selection must include all kinds of habitats where the species was found. Therefore, populations were chosen from different eco-geographic units of the second-order (EGUs) according to Meynen and Schmithüsen (1959) to capture the genetic variation of adaptive traits. EGUs represent the regions with specific abiotic (climatic, geomorphologic, geologic, hydrologic, and soil conditions) and biotic features (flora and fauna). These geofactors can have considerable influence on the number and composition of secondary metabolites and on the organic compounds (Cirak et al., 2012; Forwick, Wunder, Wingender, Mösel, & Schnabl, 2003; Ramakrishna & Ravishankar, 2011; Szakiel, Pączkowski, & Henry, 2011; Zlatić & Stanković, 2017).

(b) In some cases, the data from the agencies included possible immediate threats in the comments field of the database excerpts. Those populations were not taken into account, as the risk of these becoming extinct in the near future was too high. (c) The populations should have at least a population size of 30 individuals. (d) Also, if possible, populations existing in nature reserves (NRs) were selected. These sites already provide the infrastructure that can be used to improve the conservation of the CWR target taxon. In comparison to areas without a conservation status, NRs can sustain a genetic reserve for a more extended period.

Permission from authorities and property owners was obtained. The sites were visited in the year 2015 in order to assess the suitability of the location and the conservation status of the occurrence. In some cases, in Bavaria and Mecklenburg-West Pomerania, current monitoring data already existed and was used for further assessment. The collected data were stored in the GE-Sell database available online at [http://vm323.rz.uos.de/mapportal/pages/auswahl\\_gesell.php](http://vm323.rz.uos.de/mapportal/pages/auswahl_gesell.php).

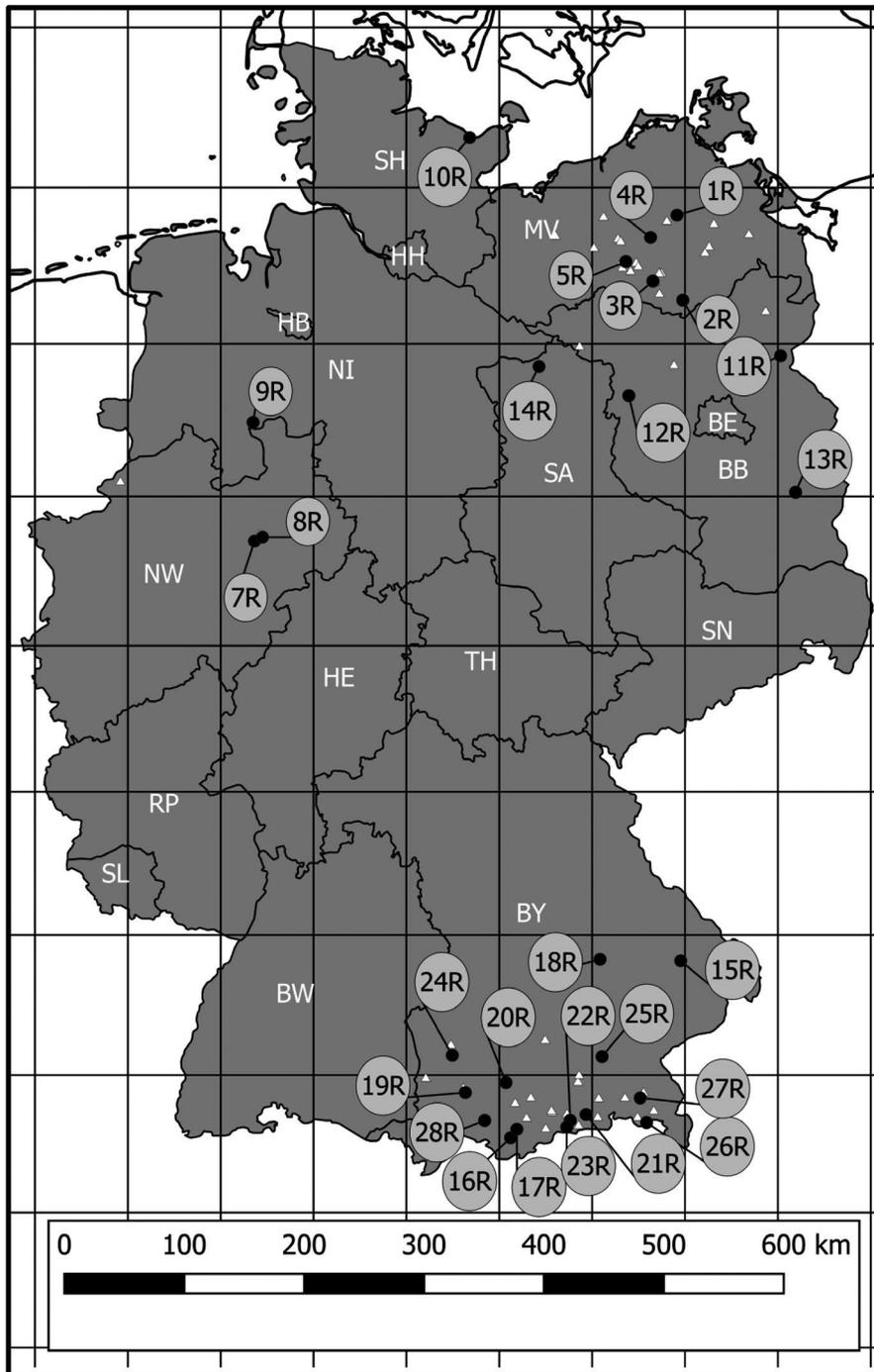
The comparison of the first assessment with the data from the EAs indicated annual variations in population sizes. Locations with high population sizes were preferred to avoid traveling to sites with temporarily small population sizes. If the preliminary assessment in 2015, or the second visit in 2016 revealed immediate threats, the populations were not taken into account. Out of the confirmed occurrences, 27 populations were selected for sampling and genetic analysis in 2016 (Figure 1). The selected populations were located in Bavaria (BY; 15R- 28R), Brandenburg (BB; 11R- 13R), Mecklenburg-West Pomerania (MV; 1R- 5R), Lower Saxony (NI; 9R), North Rhine-Westfalia (NRW; 7R and 8R), Schleswig-Holstein (SH; 10R), and Saxony-Anhalt (ST; 14R).

### 2.2 | Plant material and DNA extraction

Leaves from up to 30 individuals of 27 *H. repens* populations (Table 1) were collected (Brown & Marshall, 1995). If a population size was lower than 50 individuals, the number of sampled individuals was reduced (for the numbers of analyses samples see Table 3). Overall, 715 individuals were analyzed. The material was collected along a grid with a minimum distance of two meters, to avoid sampling from the same individual or plants with a high degree of kinship. The material was dried using silica gel and later used for the DNA isolation. Total genomic DNA was isolated using the InnuPREP Plant DNA Kit (Analytic Jena AG). As secondary metabolites inhibited the PCR, the protocol from the manufacturer was altered. After the incubation at 65°C for 30 min, 60 µl of Sorbent was added from the Diamond DNA Plant Kit (Diamond DNA), mixed on a shaker and centrifuged for 5 min on ca. 13,226 x g. If this action was performed after the final DNA elution, it resulted in the loss of the DNA (personal observation). The supernatant was then used in all further stages according to the instructions of the manufacturer. Sorbent is activated carbon with a high adsorption capacity. As it does not bind the DNA, it is therefore ideal for removing metabolites which potentially inhibit PCR reactions (for more information see the Federal Institute of Industrial Property, IPS Ru#1545641425588). Isolated DNA was diluted 1:20 and then used directly for PCR amplification.

### 2.3 | Primer design

The company TraitGenetics GmbH performed the design and construction of the forty-nine genomic SSR primer, based on the sequenced nuclear genome of *H. repens*. All microsatellites were



**FIGURE 1** Provenance of the 27 analyzed German populations of *Helosciadium repens*. Black dots: analyzed populations; population IDs correspond with the Laboratory IDs in Table 1; white triangles: preliminary assessed and confirmed populations in 2015; white letters = Federal States of Germany (BB, Brandenburg; BE, Berlin; BW, Baden Wuerttemberg; BY, Bavaria; HB, Bremen; HE, Hesse; HH, Hamburg; MV, Mecklenburg–West Pomerania; NI, Lower Saxony; NW, North Rhine–Westphalia; RP, Rheinland–Pfalz; SA, Sachsen Anhalt; SH, Schleswig–Holstein; SL, Saarland; SN, Saxony; TH, Thuringia); scale bar at equatorial scale; Pseudo–Mercator Projection

repeats of dinucleotides of various lengths. Forward primers of all sets were labeled with fluorescence dyes HEX or FAM (for primer sequences see Table 2). The markers were neutral and not subjected to any evolutionary constraint.

## 2.4 | SSR amplification

A test sample set was designed based on three populations (1R, 2R, and 9R). From each population, ten individuals were used. Microsatellite amplification was carried out for all 49 primer sets in a final volume of 20  $\mu$ l, containing 1  $\mu$ l DMSO, 2  $\mu$ l 10 $\times$

reaction buffer B (Solis BioDyne), 1.6  $\mu$ l MgCl<sub>2</sub> (25 mM), 0.4 dNTP mix (10 mM), 0.6  $\mu$ l of each primer (0.3  $\mu$ M), 1  $\mu$ l DNA, and 0.1  $\mu$ l FIREPol Taq-polymerase (Solis BioDyne). PCRs were carried out under the following touchdown PCR conditions for all loci: 94°5'30", 56°45", 72°1' [94°30", 55.5°45", 72°1']<sub>6</sub> (lowering the annealing temperature by 0.5°C every cycle) [94°30", 52°45", 72°1']<sub>31</sub> 72°10', 12°5'. Samples which failed in the first run were rerun using the 10  $\mu$ l Biozym red HS Taq master mix (Biozym Scientific GmbH), 0.6  $\mu$ l of each primer (0.3  $\mu$ M), 1  $\mu$ l DNA in a final volume of 20  $\mu$ l. PCR products were checked on an agarose gel before being sent to TraitGenics for fragment analysis. The primer test revealed that eleven out of 49 SSR primer sets produce

TABLE 1 Provenance of 27 analyzed German populations of *Helosciadium repens* and arguments for or against a nomination as a most appropriate wild population

Laboratory ID	GE-Sell ID	Location	EGU	Arguments for or against the nomination as MAWPs
1R	MV-GC-20120912-1400	Mecklenburg-West Pomerania: Demmin, Lake Kummerow, Camping Ground	Rückland der mecklenburgischen Seenplatte)	The best representative in quality standards for a genetic reserve within the EGU; camping ground suits best for public relations
2R	MV-WWR-20150806-1430	Mecklenburg-West Pomerania: Mecklenburg-Strelitz, Wesenberg	Mecklenburgische Seenplatte	3R and 5R are the better representatives in quality standards for a genetic reserve within the EGU
3R	MV-GS-20150928-0930	Mecklenburg-West Pomerania: Lake Müritz	Mecklenburgische Seenplatte	Biggest population in Mecklenburg-West Pomerania
4R	MV-MS-20141030-1400	Mecklenburg-West Pomerania: Lake Malchin	Rückland der mecklenburgischen Seenplatte	Small population; 1R is the best representative in quality standards for a genetic reserve within the EGU
5R	MV-DS-20131029-1030	Mecklenburg-West Pomerania: Lake Müritz, Alt Schwerin	Mecklenburgische Seenplatte	The population is located in a wildlife reserve, already in focus of nature conservation foundation, which owns the site
7R	NRW-LP-20150819-0945	North Rhine-Westphalia: Soest, Lippstadt, Lake Margareten	Westfälische Tieflandbucht	Small area, 8R is the best representative in quality standards for a genetic reserve within the EGU
8R	NRW-DB-20150818-1831	North Rhine-Westphalia: Paderborn, Delbrück	Westfälische Tieflandbucht	The best representative within the EGU
9R	NI-OM-20150812-0955	Lower Saxony: Diepholz, Hüde, Ochsenmoor	Dümmer-Geestniederung	The only representative in this EGU; excellent collaboration with the local conservationists in charge; management plan already exist
10R	SH-TIV-20150902-0900/0910/0920	Schleswig-Holstein: Plön, Blekendorf	Schleswig-Holsteinisches Hügelland	Excellent collaboration to the local conservationists in charge; already in focus of nature conservation foundation, which owns the site
11R	Bbg-PA-20150723-0702	Brandenburg: Barnim, Parsteinsee	Rückland der mecklenburgischen Seenplatte	Low vitality, 1R is the best representative in quality standards for a genetic reserve within the EGU
12R	Bbg-SE-20150723-1634	Brandenburg: Seeblick	Elbtalniederung	Only representative in this EGU; high numbers of individuals
13R	Bbg-JE-20150721-1018	Brandenburg: Dahme-Spreewald, Schwielochsee	Ostbrandenburgisches Heide- und Seengebiet	Only representative in this EGU; large and vital population
14R	ST-KRAAT-20130816-1119	Saxony-Anhalt: Altmarkkreis Salzwedel, near Arendsee	Altmark	Only representative, excellent collaboration to the local conservationists in charge; the local group is highly interested
15R	BY-DEG_FISC-20151024-1001	Bavaria: Deggendorf	Unterbayerisches Hügelland	Small population
16R	BY-GAP_FARC-20151021-1004	Bavaria: Garmisch-Partenkirchen, Farchant	Schwäbisch-Oberbayerische Voralpen	Eutrophication, 22R is the best representative in quality standards for a genetic reserve within the EGU
17R	BY-GAP_ESCH-20160903-1096	Bavaria: Garmisch-Partenkirchen, Eschenlohe	Schwäbisch-Oberbayerische Voralpen	Low vitality, small population, 22R is the best representative in quality standards for a genetic reserve within the EGU

(Continues)

TABLE 1 (Continued)

Laboratory ID	GE-Sell ID	Location	EGU	Arguments for or against the nomination as MAWPs
18R	<b>BY-KEH_NIED-20150908-1005</b>	Bavaria: Kelheim, Langquaid	Unterbayerisches Hügelland	Represents the whole composition of the analyzed populations the best
19R	BY-KF_KAUJF-20150814-1012	Bavaria: Kaufbeuren	Voralpines Hügel- und Moorland	Small population; eutrophication, 27R is the best representative in quality standards for a genetic reserve within the EGU
20R	BY-LL_BISC-20160828-1022	Bavaria: Landsberg am Lech, Dießen am Ammersee	Voralpines Hügel- und Moorland	27R is the best representative in quality standards for a genetic reserve within the EGU
21R	BY-MB_TRAC-20150811-1002	Bavaria: Miesbach, Fischbachau	Schwäbisch-Oberbayerische Voralpen	Medium vitality, eutrophication, 22R is the best representative in quality standards for a genetic reserve within the EGU
22R	<b>BY-MB_TRIN_20150802-1003</b>	Bavaria: Miesbach, Kreuth	Schwäbisch-Oberbayerische Voralpen	Represents the uniqueness population within this composition; large plain aquatic population
23R	BY-MB_WILD-20150731-1001	Bavaria: Miesbach, Kreuth	Schwäbisch-Oberbayerische Voralpen	Medium vitality, 22R is the best representative in quality standards for a genetic reserve within the EGU
24R	<b>BY-MN_SALG-20150804-1019</b>	Bavaria: Unterallgäu, Salgen	Donau-Iller-Lech-Platten	Single representative in this EGU; high numbers of individuals
25R	BY-MUE_MARS-20150829-1027	Bavaria: Mühldorf a. Inn, Maitenbeth	Voralpines Hügel- und Moorland	Medium vitality, 27R is the best representative in quality standards for a genetic reserve within the EGU
26R	<b>BY-TS_WINK-20150812-1014</b>	Bavaria: Traunstein, Reit im Winkl	Nördliche Kalkhochalpen	Single representative in this EGU; high numbers of individuals
27R	<b>BY-TS_WINK-20151114-1001</b>	Bavaria: Traunstein, Übersee	Voralpines Hügel- und Moorland	A large population within a wildlife reserve
28R	BY-WM_SAUW-20160907-1124	Bavaria: Weilheim-Schongau, Prem	Voralpines Hügel- und Moorland	Small population, 27R is the best representative in quality standards for a genetic reserve within the EGU

Note: Laboratory ID = population work ID, GE-Sell ID = reference IDs used in the project GE-Sell (bold letters- MAWP), location = locations in Germany (Federal State: description of the location), EGU = eco-geographic units (order two) based on Meynen and Schmithüsen (1959).

**TABLE 2** SSR primers sets used in the analysis of 27 populations of *H. repens* in Germany, assessed with six microsatellites

Primer ID	Dye	F-primer	R-Primer
ANM0057	FAM	AATATTATTGATTGGAGTGCCTTT	TGAGGTTGTAATAGGCTATCATCAGT
ANM0066	HEX	TGGCAGCCTGGATAACTACC	AGTAAGGAGAAGTAAGTGAACAAGAGA
ANM0077	HEX	AATACATACATACATGCCTTCACTAAG	CAATAAGTGCTTGAGAATCTAATAGG
ANM0079	HEX	AAGCCACATAGCAAACCTGTC	CGTGCAAAGTTGTGGTGTCT
AXM0081	FAM	GGGAGTGATGGTAGGAGAGTAGAA	TGAGAATCAATTAATTTGGTGAAGG
AXM0083	FAM	TTGCCACTTTCATTACATCTTCA	AGAACATCCAAGTTATGCTGACAA
AXM0087	FAM	TCCAACCTAATCCATCTCTACACA	AAAGAGATACACAGTTATCGAGGAG
AXM0090	FAM	TCAAGATGGCCTTCTCAAGT	AAAGAAGGATACTGACCAGGCTT
AXM0091	FAM	ACGTAGAAACCTGCACCCAA	CCCTTTCTTCTCCCTGATG
AXM0105	HEX	TCGTAGGGAGACCATGTAGCTT	AATGGGCCAACCCAAAGT
AXM0108	HEX	GCTAAATTTACGGTTGGTTCTCT	CTAATAGTTAACCCATAATTTGGAGAA

Note: Primer ID = identification code of the primer sets (bold letters- primer sets used in the final analysis), dye = fluorescence marker of the forward primer (HEX- Hexachloro-Fluorescein, FAM- 5(6)-Carboxyfluorescein), F-primer = forward primer sequence, R-primer = reverse primer sequence.

suitable products for further analysis. However, only six of these amplified regions across all populations successfully and were used in the further analysis. Individuals which failed to amplify in one or more primer sets were excluded.

The software Genemapper v5.0 (Thermal Fischer Scientific Inc.) was used to evaluate the chromatograms by identifying all microsatellite alleles and their respective sizes. Each call was checked manually and corrected if necessary. Primer sets which successfully amplified polymorphic products in all test populations were used to analyze all 27 populations.

## 2.5 | Data analysis

Based on previous exclusion, out of the 763 collected individuals, 715 were used in the analysis (Table 3). SAS ProcAllele procedure was used to test the Hardy–Weinberg Principle (HWP) and calculate allele frequencies, polymorphic information index (PIC), observed ( $H_o$ ), and expected heterozygosity ( $H_e$ ) using  $10^4$  permutations and 5,000 bootstraps pseudo-replicates. The SSR data was converted manually into a genepop format and loaded in R using the package *adegenet* for further analyses (Jombart & Collins, 2015). Private alleles (alleles unique to a specific population) were counted with the function *private\_alleles* from the R package *poppr* 2.8.1 (Kamvar, Tabima, & Grünwald, 2014), and rare alleles, at a frequency  $\leq 0.05$ , were recovered from the SAS output data. Rare and private alleles were related to the sample size of the population. Allelic richness was measured with rarefaction using the *allele.rich* function from the R package *PopGenReport* (Gruber & Adamack, 2014) and based on the works of Hurlbert (1971). The smallest number of individuals sampled across all combinations of populations and loci was 14. The measure of deviation from panmixia at the local scale ( $F_{IS}$ ) was calculated with the software Fstat2.9.3.2 (Goudet, 2001) and the fixation index F with GenAlEx6.51b2 (Peakall & Smouse, 2006, 2012). Tests for significance were carried out with the *geom\_signif* function using

the R package *ggplot2*. Plots and graphs were drawn using the function *ggplot* from the R package *ggplot2* (Wickham, 2016).

The measure  $\Delta$  is free of model assumptions such as the presence of large, random mating populations in the Hardy–Weinberg equilibrium (HWE; Gregorius, Gillet, & Ziehe, 2003) and ranges between 0 (no genetic distance between a pair of populations) and 1 (highest possible genetic distance between a pair of populations). The software *DifferInt* was used to calculate the complementary compositional differentiation among populations, whereby  $\Delta_j$  is the contribution of the  $j$ th population to genetic differentiation.  $\Delta_j$  is the genetic distance of the  $j$ th population to the pooled remainder (“the complement”). A population with  $\Delta_j = 0$  population represents exactly its complement, while the genetic composition of a population with  $\Delta_j = 1$  is entirely different from its complement.  $\Delta_{SD}$  quantifies the average degree to which all populations differ from their complements (Gillet, 2013). *DifferInt* calculates the complementary compositional differentiation at different levels of genetic integration: single-locus genotypes (SLG) and the multi-locus genotypes (MLG). Effects of differences among the populations' gene pools and gene association within the gene pools on differentiation were compared by two permutation analysis (Gillet, 2013;  $10^3$  random permutations).

Population structure analysis was carried out using a discriminant analysis of principal components (DAPC) implemented in the R package *adegenet* (Jombart, Devillard, & Balloux, 2010). This analysis is comparable with an analysis by the software *Structure* (Evanno, Regnaut, & Goudet, 2005). However, it does not assume random mating populations in HWE (Jombart et al., 2010). The function *find.clusters* was used to identify the number of genetic groups (hereafter  $K$ ; 50,000 iterations and five random starting centroids) and the function *optim.a.score* to find the optimal number of principal components. Additionally, another independent nonmodel approach was used to confirm the result. This method was based on the replicated nonhierarchical  $K$ -means clustering (Hartigan & Wong, 1979) using the R-script of Arrigo et al. (2010). We performed  $5 \times 10^4$

TABLE 3 Genetic diversity parameters for each of the 27 analyzed German populations of *Helosciadium repens* assessed with six microsatellites

Laboratory ID	n	MLG	SLG	A	Rare	Private	all.rich	Mean Ho	Mean He	F	F <sub>is</sub>	deltaSD	Cat	Form
1R	27	11	20	16	0	0.037	1.870	0.093	0.167	0.336	<b>0.462</b>	0.3884	N	terr
2R	28	3	18	14	0	0	1.857	0.250	0.253	-0.009	0.028	0.36	N	terr
3R	29	8	11	11	0	0	1.596	0.201	0.171	<b>0.087</b>	-0.163	0.375	N	terr
4R	7	7	15	12	0	0	1.910	0.214	0.274	<b>0.203</b>	<b>0.289</b>	0.3136	N	terr
5R	25	3	9	11	0	0	1.251	0.013	0.039	<b>0.656</b>	<b>0.667</b>	0.447	N	terr
7R	26	4	8	8	0	0	1.116	0.006	0.019	<b>0.490</b>	<b>0.667</b>	0.3064	N	terr
8R	30	7	11	11	0.033	0	1.489	0.089	0.094	<b>0.143</b>	<b>0.066</b>	0.4104	N	terr
9R	30	6	12	12	0	0	1.568	0.106	0.130	<b>0.330</b>	<b>0.206</b>	0.2798	N	terr
10R	29	6	7	7	0	0.034	1.109	0.023	0.021	-0.074	-0.057	0.3798	N	terr
11R	30	4	10	10	0	0.033	1.176	0.028	0.027	-0.026	-0.01	0.2952	N	terr
12R	26	3	8	8	0	0	1.140	0.026	0.024	-0.040	-0.031	0.3409	N	terr
13R	30	1	6	6	0	0	1.000	0.000	0.000	<b>0.470</b>	NA	0.4394	N	terr
14R	15	5	10	11	0	0.200	1.471	0.044	0.083	<b>0.470</b>	<b>0.491</b>	0.3598	N	terr
15R	29	27	31	19	0.034	0	2.662	0.448	0.501	<b>0.095</b>	<b>0.122</b>	0.2829	S	terr
16R	30	5	10	9	0	0	1.443	0.178	0.155	<b>0.137</b>	-0.13	0.3155	S	aqu
17R	28	23	22	16	0	0	2.177	0.274	0.352	<b>0.211</b>	<b>0.24</b>	0.3914	S	terr
18R	27	22	18	17	0.037	0	2.051	0.321	0.310	-0.012	-0.016	0.2498	S	terr
19R	30	5	9	10	0	0	1.631	0.383	0.260	-0.241	-0.464	0.3617	S	aqu
20R	29	6	10	10	0	0	1.409	0.172	0.178	<b>0.013</b>	<b>0.047</b>	0.2966	S	aqu
21R	30	10	14	13	0.067	0	1.920	0.339	0.284	-0.184	-0.177	0.4754	S	aqu
22R	29	12	15	14	0	0.034	1.784	0.247	0.266	<b>0.051</b>	<b>0.089</b>	0.4902	S	aqu
23R	30	2	7	7	0	0	1.037	0.006	0.005	-0.017	0	0.4161	S	aqu
24R	29	8	11	12	0	0	1.515	0.310	0.191	-0.329	-0.617	0.3136	S	aqu
25R	28	7	12	13	0	0	1.868	0.512	0.301	-0.505	-0.69	0.3845	S	aqu
26R	29	29	36	21	0	0.069	3.152	0.420	0.586	<b>0.248</b>	<b>0.301</b>	0.342	S	terr
27R	26	20	30	18	0.038	0	2.599	0.340	0.439	<b>0.350</b>	<b>0.245</b>	0.268	S	terr
28R	9	8	14	14	0	0.333	2.068	0.296	0.320	<b>0.094</b>	<b>0.132</b>	0.3732	S	terr

Note: Laboratory ID = population work IDs corresponding with the Laboratory IDs in Table 1 (bold = MAWPs), n = sample size in the analysis, MLG = numbers of multi-locus genotypes, SLG = numbers of single-locus genotypes, A = numbers of alleles, rare = number of rare alleles per individual, private = number of rare alleles per individual, all.rich = average allelic richness, mean H<sub>o</sub> = mean observed heterozygosity, mean H<sub>e</sub> = mean expected heterozygosity, F = Fixation Index (bold- positive values), F<sub>is</sub> = F<sub>is</sub> Index after Weir and Cockerham (1984) (bold- positive values), deltaSD = compositional differentiation at genotype level, cat = category (S = Southern populations, N = Northern populations), form = ecological form (terr = terrestrial; aqu = aquatic).

independent runs (starting from random points) for each of the assumed groups between two and 30. The intergroup inertia was recorded as a proxy of clustering accuracy, and the delta  $K$  values were calculated (Evanno et al., 2005) using the method adopted by Arrigo et al. (2010). The values with the highest delta  $K$  were considered the optimal number of groups in the data. Pie charts showed the percentage of individuals assigned to a genetic group. They were drawn using the function *pie* from the R package *graphics* (Becker, Chambers, & Wilks, 1988; Cleveland, 1994). All packages were used in RStudio 1.0.153 (R Core Team, 2017; RStudio Team, 2016).

Maps were drawn with QGIS-2.8.1-Wien (QGIS Development Team, 2009) with a pseudo-Mercator projection. Natural Earth (www.naturalearthdata.com) provided the free vector and raster map data.

## 2.6 | Selection criteria for MAWPs

The results from DifferInt were used to guide the selection of populations for genetic reserves. As means for conservation are always limited, the procedure was started with the population which had the lowest and highest  $\Delta_j$  at the gene pool level. (a) In every EGU represented in the set of 27 sites, at least one genetic reserve should be established to maximize the chance of capturing adaptive trait variation. To this end, one population was selected from each EGU. (b) Large population size was preferred over smaller population size. (c) As genetic reserve management relies on the support of local nature conservation agencies, other institutional stakeholders and volunteers, organizational and social aspects were also taken into account. (d) If the collectors found an immediate threat during the collection phase in 2016, the population in question was not considered as a MAWP. (e) Populations with an existing management plan, regardless of their conservation status, were given priority.

## 3 | RESULTS

### 3.1 | Distribution

Of the 78 preliminary assessed sites, 59 contained *H. repens* populations. The largest population in MV was 3R with a distribution area exceeding 12,000 m<sup>2</sup>. *Helosciadium repens* is often found in patches rather than in continuous populations. Considering this, 12R with 400 m<sup>2</sup> of a populated area was the largest population in the whole Northern area. In BY, the largest population was 22R with a population area of 350 m<sup>2</sup>, distributed over an area of 89,000 m<sup>2</sup>.

### 3.2 | SSR analysis

The numbers of alleles per locus ranged from four to nine (AXM0105 and AXM0081, respectively), and the numbers of alleles per population ranged from six to 21 (13R and 26R, respectively). The PIC ranged

between 0.3646 (AXM0105) and 0.5802 (AXM0090). Out of the 38 distinct alleles, 12 alleles were private and three were rare (Table 3). The  $H_o$  and the  $H_e$  of each locus ranged from 0.1748 to 0.2755 (AXM0087 and AXM0105) and 0.2756 to 0.6389 (AXM0087 and AXM090), respectively. Twenty-two populations were not in the HWE ( $p < .05$ ; Table S1). From the 162 cases (six primer sets  $\times$  27 populations), a significant deviation from the HWE was found in 49, and in 54 cases the markers were monomorphic. The only populations that were in HWE were 10R, 11R, 12R, 13R, and 23R (Table S1). In these populations, one to three markers had heterozygote genotypes and in 13R all the markers were homozygote. The  $F_{IS}$  Index ranged from  $-0.617$  (24R) to  $0.667$  (5R and 7R; Table 3). Out of the 27 occurrences, ten showed an excess of heterozygosity, while 15 showed an excess of homozygosity (Table 3, excess of homozygosity in bold in the  $F_{IS}$  column). According to the  $F_{IS}$  Index, population 23R showed panmixia (Table 3). The fixation index  $F$  varied between  $-0.505$  (25R) and  $0.656$  (5R). Out of the 27 populations, 16 exhibited inbreeding (Table 3). Ten populations showed an excess of heterozygosity (Table 3, excess of homozygosity in bold in the  $F$  column). The allelic richness and the amount of MLGs were significantly higher among the BY populations (S) in comparison to the Northern populations (N) ( $p < .05$ ; Figure 2, Table 3). However, the amount of SLG, rare, and private alleles and the  $F_{IS}$  Index values were not significantly different (data not shown).

### 3.3 | Complementary compositional differentiation

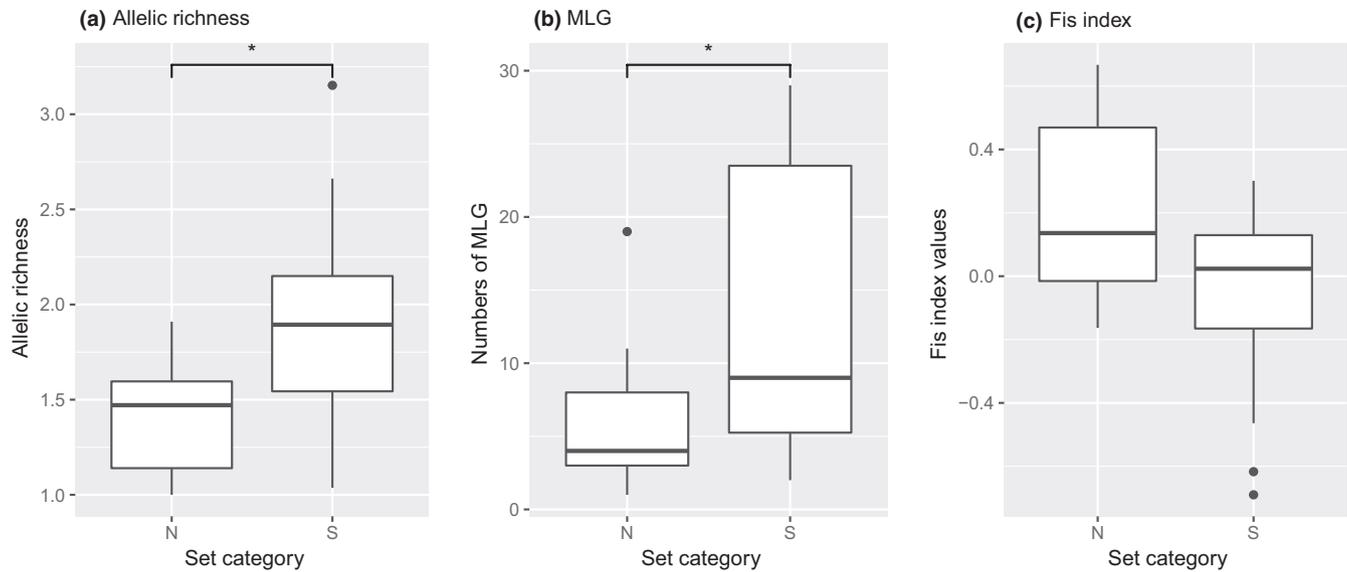
The numbers of SLG spanned from eight to 16 per locus (ANM0079 and AXM0105 with the lowest and AXM0090 with the highest count) and ranged from six to 36 (13R and 26R, respectively) in populations. The MLG spanned from one to 29 (13R and 23R with the lowest and 26R with the highest count). Within the whole data set (715 individuals and six markers), 68 SLG and 235 MLG were identified. Within populations, some MLGs were found to be duplicated ranging from two to 30 times. Population 13R was composed of only one MLG (Table 3).

The mean compositional differentiation at the genotype level was  $\Delta_{SD} = 0.3455$  and increased to  $\Delta_{SD} = 0.3598$  at mean SLG and  $\Delta_{SD} = 0.3691$  at the MLG level. At the mean SLG and the MLG level, the  $\Delta_{SD}$ -values observed were higher than 95% of all  $\Delta_{SD}$ -values generated by the first permutation analysis. At all levels of integration, the  $\Delta_{SD}$ -values were higher than 95% of all  $\Delta_{SD}$ -values generated by second permutation analysis.

22R was identified as the population with the highest  $\Delta_{SD}$ . Thus, it represented the population which differs most from the complement. The population 18R with the lowest  $\Delta_{SD}$  was the population which represents the whole complement the best (Figure 3, Table 3).

### 3.4 | Discriminant analysis of principal components

The Bayesian information criterion (BIC) versus number-of-clusters plot showed no clear indication of the "true  $K$ " (data not shown). In



**FIGURE 2** Comparison of the 27 analyzed German populations of *Helosciadium repens* assessed with six microsatellites. (a) Allelic richness (b) multi-locus genotype (MLG) (c)  $F_{IS}$  Index values. N: northern populations (1R–14R), S: southern populations (15R–28R), asterisks indicate significance at the 0.05 level

the  $\Delta K$  versus numbers of groups ( $K$ ) plot, the value with the highest  $\Delta K$  was at  $K = 2$ . However, a  $K$  between two and six was also considered possible (Figure S1). Therefore, we performed the DAPC with  $K$  equals two, four, and six. Populations were associated with the cluster with the highest obtained cluster assignment.

For  $K = 2$  the DAPC showed a division of N and S populations (data not shown). Only one population (16R from BY) did not coincide with its geographical distribution (with 83% of the individuals affiliated with the Northern cluster). Three populations (1R, 3R, and 4R) also had some individuals (<14%) affiliated with the Southern cluster. In the Southern cluster, there were eight populations with individuals associated with the Northern cluster (between 3% and 48%). For  $K = 4$  and  $K = 6$ , the DAPC revealed similar, but more detailed clustering, compared with  $K = 2$  (data for  $K = 4$  not shown). However, with  $K = 6$ , only one population did not coincide with its geographical distribution (16R). Therefore,  $K = 6$  was regarded to be the optimal number of clusters (Figure 4; for exact numbers, see Table S2).

Most clusters can be correlated with specific geographical regions. Populations from MV (1R, 2R, 3R, 4R, and 5R) and Western BB (12R) were allocated explicitly to cluster three. The rest of the North German populations were mostly linked to cluster five. Some individuals in a population were not assigned to the same cluster as the rest of the population (7% on average). When they are compared to the N populations, the S populations are more heterogeneous. Nevertheless, some populations from a specific region were allocated to a particular cluster (such as Western Bavaria populations—19R, 20R, 24R, and 28R to cluster one and the central and Southern populations—18R, 23R, and 25R to cluster two) the regions which were affiliated to specific clusters were mostly overlapping (28% on average). Populations 15R, 18R, 20R, 22R, 26R, 27R, and 28R retrieved relatively high affiliation with more than one cluster.

The BY populations can be organized into three groups according to the cluster assignment: East BY with 15R, 26R 27R, central BY with 18R, 21R, 22R, 23R, 25R, and West-BY with 19R, 20R, 24R, 28R. Occurrences 16R and 20R had a high affiliation to cluster five, and 17R to cluster six. There was no correlation between the clusters and EGUs.

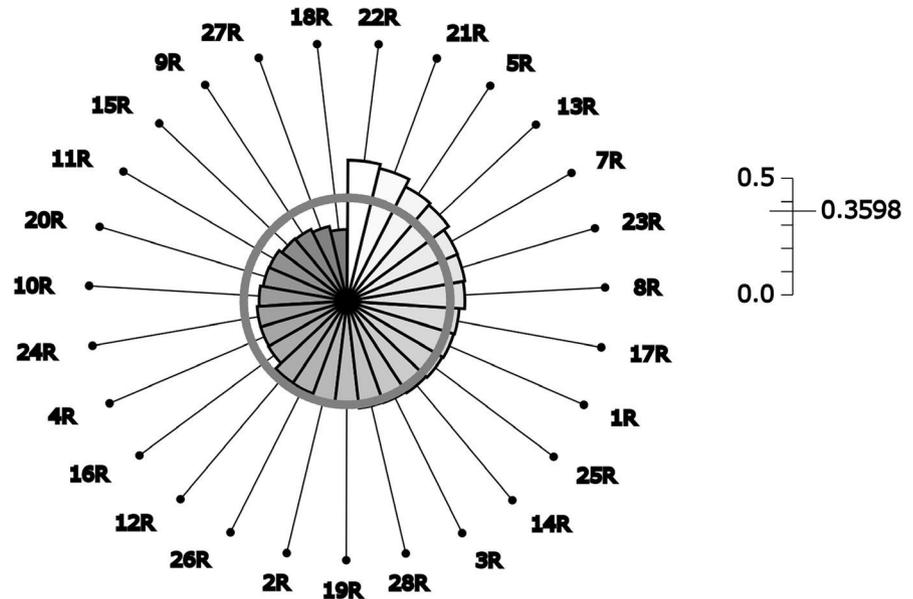
### 3.5 | Selection of MAWPs

Besides the two selected populations based on the results from DifferInt (22R and 18R), populations 1R, 3R, and 5R from MV, 8R from east Muensterland region, 9R from Lower Saxony (NI), 12R and 13R from Brandenburg (BB), and 24R, 26R, and 27R from BY were also selected as MAWPs (for justification see Table 1). Additionally, two populations were selected as complementary though suboptimal MAWPs. These were the only representatives of their EGU but had a critically low population size (14R from Saxony-Anhalt- ST) or was introduced (10R from Schleswig-Holstein- SH).

## 4 | DISCUSSION

Our study presents an analysis of genetic diversity and genetic differentiation based on a set of populations of *H. repens* sampled within the entire distribution area in Germany. The three main results derived from the analysis of 27 occurrences with six SSR markers are the following: (a) the analyzed markers show a low level of genetic variation between populations in Germany. (b) The populations are divided into Northern and Southern populations. (c) MAWPs suited to establish genetic reserves were identified and recommended.

**FIGURE 3** Snail diagram showing the differentiation of each of the 27 analyzed German populations of *Helosciadium repens* to their complement at the gene pool level. The data were generated with six microsatellites and estimated by the software DifferInt. The side length of a sector quantifies the contribution of each occurrence to the differentiation. The gray circumference represents the overall  $\Delta_{SD}$  values, which are given at the top right of the chart. Populations ID correspond with the Laboratory IDs in Table 1



#### 4.1 | Low level of genetic variation

The first permutation analysis randomly permutes the alleles among the individuals within each population. In a panmictic population, one would expect that gene association in individuals do this independent of the allelic type at each locus and type at a given level of integration (Gillet, 2013). If this hypothesis were correct, the  $\Delta_{SD}$ -values of the integration level SLG and MLG would be within the 95% confidence interval of all  $\Delta_{SD}$ -values generated by the first permutation analysis (Gillet, 2013). However, from the data generated by the SSR analysis, this hypothesis must be rejected. Tests for HWP also indicated nonrandom mating in 22 of the analyzed populations (Table S1).

One explanation for the indication of nonrandom mating revealed by the markers could be explained with runner growth. It is namely *H. repens* primary strategy to colonize open areas. However, the method of collecting material was designed to avoid sampling from the same individual or plants with a high degree of kinship. Another, and yet more likely explanation would be self-fertilization or preferential mating within half- or full-sib families. The high number of MLG duplications within populations and the excess of homozygotes shown in 14 populations by the  $F_{IS}$  and F-Index seem to confirm this interpretation (Table 3). *Helosciadium repens* does produce high amounts of seeds. A prime example was population 13R, which is composed of only one MLG. As 80% of the individuals observed in 2016 were flowering, it is probably not a clonal population.

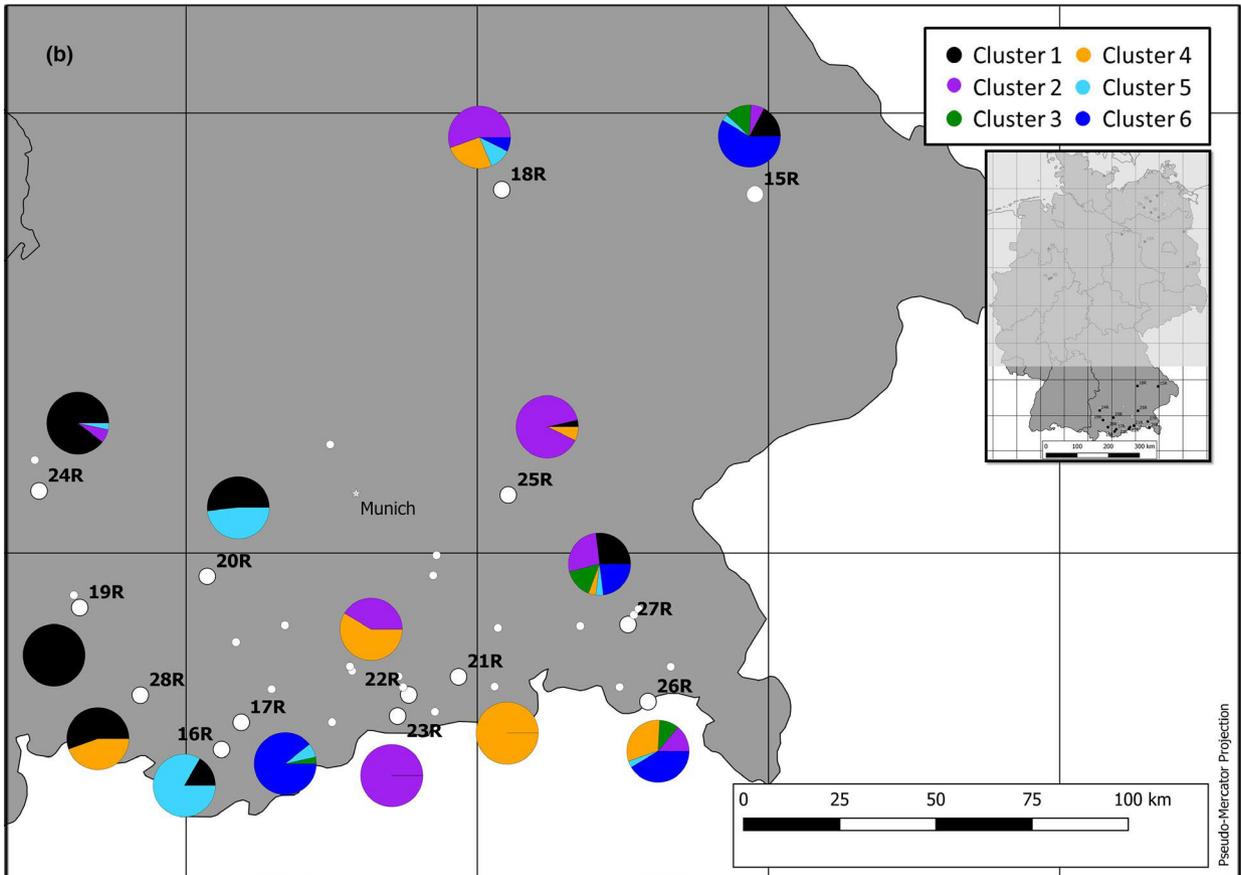
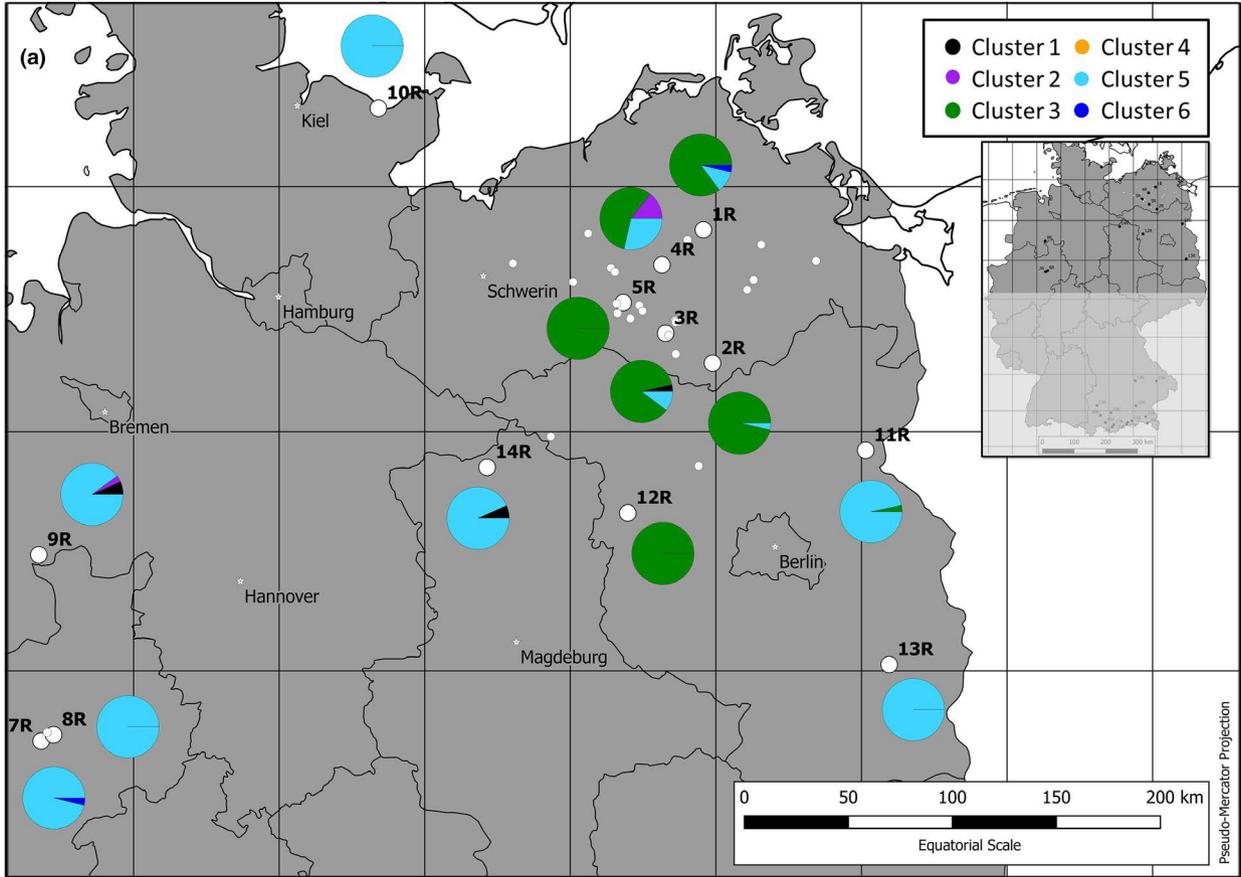
In 12 populations either the  $F_{IS}$  or F-Index, or both values, were negative (Table 3). Small populations, or low numbers of reproducible individuals, overdominance, self-incompatibility (SI) or asexual propagation are common explanations (Stoeckel et al., 2006). As the markers used were neutral and *H. repens* is not known for possessing any self-incompatibility systems, the most likely explanation would be asexual reproduction. Almost all aquatic populations were among

those 12 cases (except 22R). Schossau (2000, cited in Hacker et al., 2003) said that aquatic and semi-aquatic populations tend to prefer vegetative growth. Nearly, all aquatic occurrences tend not to produce flowers. However, our study did not find any significant difference in the allelic richness or the mean  $\Delta_{SD}$ -values between aquatic and terrestrial populations (Table 3).

The second permutation analysis randomly permutes the individuals with their genetic types among the populations. The forces that associate individuals with populations do this independently of their genetic type at a given level of integration if the observed  $\Delta_{SD}$ -values are within a 95% confidence interval (Gillet, 2013). This hypothesis must be rejected due to differences among the gene pools of the 27 occurrences that were not randomly distributed. In other words, there is possibly no migration between the populations.

#### 4.2 | A North-South split of the German distribution area

The comparison of the allelic richness and MLG between the North and the South revealed that S populations tend to be more diverse (Figure 2). This distinction is also visible in the DAPC map (Figure 4). The S populations (mostly the South-Eastern) are part of various genetic clusters compared with the N populations. One plausible explanation for this difference in diversity can be given by assuming that *H. repens* refugia during multiple glacial periods was somewhere in the South of Europe (possibly South-East). Spalik, Banasiak, Feist, and Downie (2014) estimated that *H. repens* diverged approximately two million years ago and, therefore, has been influenced by glacial and interglacial periods. During the recolonization of the Northern parts after the last glacial maximum, diversity was lost due to bottleneck effects (Hewitt, 1996, 1999). Similar events are also known for *Calluna vulgaris* (Mahy,



**FIGURE 4** Discriminant Analysis of Principal Components (DAPC) with  $K = 6$  clusters of all 27 analyzed German populations of *Helosciadium repens* based on the results of six microsatellites. (a) Northern part of Germany; (b) Southern part of Germany. Pie charts showing the percentage of individuals assigned to a cluster; white dots-analyzed populations, populations ID correspond with the Laboratory IDs in Table 1; small white dots-preliminarily assessed populations in 2015

Vekemans, Jacquemart, & Sloover, 1997), various Bryophytes (Cronberg, 2000), *Abies alba* (Konnert & Bergmann, 1995), *Allium ursinum* (Herden, Neuffer, & Friesen, 2012) and many other European species. To prove this assumption, a broader study on a European scale would be necessary.

### 4.3 | Successfully identified MAWPs

Candidates for potential genetic reserves were successfully identified using SSR markers, previously collected populations and site data. With the lowest  $\Delta_{SD}$  value, the population in BY, Kellheim (18R) resembles the genetic diversity of all remaining 26 populations better than any other (Figure 3, Table 3). The population from BY, Miesbach (22R) had the highest  $\Delta_{SD}$  value, which means it differed the most from its complement. One can interpret this high differentiation as specific adaptation to this site. The microsatellites are well suited to obtaining insight into genetic variation, but they cannot detect adaptive trait variations. Therefore, we increased the chance to capture adaptive trait variations by also choosing populations from different EGUs. The 27 populations were present in 13 different EGUs. The current selection of the MAWPs had representatives in all 13 EGUs (Table 1). A large population size increases the chance of sustaining long-term population viability and is one of the key quality standards proposed by Iriondo et al. (2012). The largest Northern populations based on distribution over a specified area (MV, Großer Schwerin- 3R) and the largest occupied area (BB, Seeblick- 12R) were included. The population in BY, Miesbach (22R) was also included as constituent part of the MAWP candidates because to its size, and due to the fact that it is the largest analyzed population in Southern Germany. At the time of determining the areas the taxonomical status both forms take (aquatic and terrestrial) was still not clear. If both had been mentioned in the same source, the authors have always addressed them independently (Casper & Krausch, 1981; Hacker et al., 2003; NLWKN, 2011; Voightländer & Mohr, 2008). Therefore, the set of MAWPs from BY also include two aquatic populations, which represent 40% of the BY candidates. Recently, Herden and Friesen (2019) compared both forms genetically and morphologically and found no evidence for taxonomic division.

Due to limited funding, the selection of MAWPs also needs to be centered on feasibility and cost-effectiveness. Naidoo et al. (2006) pointed out the importance of economic costs in conservation projects. By prioritizing sites on already protected areas and areas with substantial support from local organizations (governmental or non-governmental), the acquisition and management costs (Naidoo et al., 2006) were minimized. Management plans and facilities already exist in NRs and may only need to be changed slightly for the benefit of

the target taxon. Also, the long-term persistence of a genetic reserve within protected areas is far more likely due to the laws and regulations to which they are subject. As a genetic reserve has no legal power and is extremely dependent upon volunteer work, social aspects (such as the interests of the landowners) are considerably important, and scientific reasoning has to take second place. However, rejection of a particular population does not mean that they are irrelevant or too insignificant to be included in future studies.

## 5 | CONCLUSIONS

Our study showed that the eight-step process proposed by Frese, Bönisch, Herden, et al. (2018) (for an English version see also Frese, Bönisch, Nachtigall, Bönisch, Nachtigall, & Schirmak, 2018) is well suited for identifying MAWPs for establishing genetic reserves. Based on this study, the first European genetic reserves for *H. repens* were established in June 2019 (3R and 12R). In Germany, the genetic reserve has no legal status. Long-term success is highly dependent on the support and active collaboration of local people. *Helosciadium repens* patchy population structure should be considered when collecting seeds for storage in gene banks. Seeds from every MAWP should be collected for ex situ preservation of genetic diversity in gene banks. We recommend making the samples available for plant breeders and conservationists, as the sustainable use of wild populations is an argument toward investing in further conservation activities. The seeds can be stored in the WEL Gene Bank (National Gene Bank for German Crop Wild Relative Species, Botanical Garden of Osnabrueck, Germany; see Table 1 for reference IDs).

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## CONFLICT OF INTEREST

None to declare.

## AUTHORS' CONTRIBUTION

N.F. and T.H. conceived the ideas. T.H. homogenized the data excerpts did the laboratory work and led the writing of the manuscript. M.B. organized the data excerpts, managed the first and second assessment of the sites, and together with T.H. was involved in the decision process of the MAWPs. All authors contributed critically to the draft.

## OPEN RESEARCH BADGES



This article has been awarded Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://doi.org/10.5061/dryad.rr4xgxd5c>.

## DATA AVAILABILITY STATEMENT

The data are available under <https://doi.org/10.5061/dryad.rr4xgxd5c>.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## Chapter 4

ECOTYPES OR PHENOTYPIC PLASTICITY - THE AQUATIC AND  
TERRESTRIAL FORMS OF *HELOSCIADIUM REPENS* (APIACEAE)

HERDEN, T. AND FRIESEN, N.

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## ORIGINAL RESEARCH

# Ecotypes or phenotypic plasticity—The aquatic and terrestrial forms of *Helosciadium repens* (Apiaceae)

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**Abstract**

- Morphological and ecological differences of two forms of *Helosciadium repens* are known and described in the literature: aquatic and terrestrial. However, their taxonomic status is currently unknown. The question whether they are genotypically adapted to specific environmental conditions or are those differences a result of phenotypic plasticity is addressed in this study.
- SSR and ISSR data were used to uncover genotypic differences. Data from drought stress experiments (system water content and relative water content of leaves) were used to evaluate the response to water as an environmental factor. The stomatal index of both forms grown under different water treatments was analyzed.
- The principal component analysis of the ISSR data revealed no clustering that would correspond with ecotypes. The diversity parameters of the SSR data showed no significant differences. The aquatic populations showed a tendency toward heterozygosity, while the terrestrial ones showed a bias toward homozygosity. Both forms responded similarly to the changes in water availability, with newly produced leaves after drought stress that were better adapted to repeated drought stress. Stomatal indices were higher in plants from aquatic habitats, but these differences disappeared when the plants were grown in soil.
- The observed responses indicate that the differences between forms are due to phenotypic plasticity.

**KEYWORDS**creeping marshwort, ecotypes, fingerprinting, genetic differentiation, *Helosciadium repens*, phenotypic plasticity

## 1 | INTRODUCTION

Most organisms exhibit different phenotypes in response to different environmental factors (Xue & Leibler, 2018). Heterophylly in *Neobeckia aquatica* (Eaton) Greene in response to the temperature and submergence (Amano et al., 2015) and *Arabidopsis thaliana* as a response to light (Mishra et al., 2012) are only two of many examples that can be found in the plant kingdom. Even metabolic

changes in the form of carbon fixation can occur such as in the case of *Mesembryanthemum crystallinum* L. (Tallman et al., 1997). These responses are all considered to be evolutionary strategies for adapting to variable environments (Xue & Leibler, 2018). In some extreme cases such as in *Pinus sylvestris* L., the high spectrum of phenotype variability led to the assignment of various names currently recognized as synonyms (The Plant List, 2013). Contrarily to the characteristics, which justify taxa levels, those apparent morphological

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**FIGURE 1** Terrestrial and aquatic forms of *Helosciadium repens* (a) terrestrial form at the natural site, (b) aquatic form at the natural site, (c) leaf of terrestrial form, (d) inflorescence of the terrestrial form, and (e) leaf of the aquatic form

differences are nonpermanent and disappear when the plants grow under the same conditions.

*Helosciadium repens* (Jacq.) W.D.J. Koch (Apiaceae) (Figure 1) is a small perennial herb, growing on alternating wet pastures, littoral zones of trenches and springs (Weber, 1995), and along or in slow running streams (pers. observation). Two forms are known and described from the literature (Casper & Krausch, 1981; Hacker, Voightländer, & Russow, 2003; NLWKN, 2011; Voightländer & Mohr, 2008). The terrestrial (hereafter *Terr*) form is hemicryptophytic and grows leaves with a length between 10 and 30 cm (Oberdorfer, 1983; Schubert & Vent, 1994). Their stolons can grow to a length between 20 and 30 cm and can as such colonize open patches very quickly (Hacker et al., 2003). The flowers are arranged in an umbel and produce nectar and a schizocarp fruit which releases two seeds per flower (East, 1940; Frank & Klotz, 1990; T. Herden, M. Bönisch, & N. Friesen, unpublished data; Klotz, Kühn, & Durka, 2002; NLWKN, 2011). *Helosciadium repens* also build up soil seed banks (Burmeier & Jensen, 2008). According to the database *BIOLFLOR* (Frank & Klotz, 1990), *H. repens* can self-fertilize. They, however, cite East (1940) as a reference for this statement. Upon further investigation, we uncover that East (1940) stated that little is known about the self-fertilization in this taxon (applying to Umbelliferae) and did not mention *H. repens* at all.

The hydrophytic populations or aquatic form (hereafter *Aqu*) can be occasionally found in Southern Germany, Bavaria (pers. observation). The aquatic form tends to exhibit vegetative growth only and does not produce flowers (Casper & Krausch, 1981; Schossau 2000 cited in Hacker et al., 2003; NLWKN, 2011). They can grow leaves up to 40 cm in length (Casper & Krausch, 1981), can colonize waterbodies up to a depth of 60 cm, and their stolons can grow up to a length of 150 cm (Voightländer & Mohr, 2008). They stay immotile due to

their roots anchored on driftwood, tree roots, or other aquatic vegetation. The plants do not root in the substrate (pers. observation).

There is scarce information on the two different manifestations in the literature. However, when mentioned, authors address both appearances as different forms of the species, and do not specify what the word “forms” means in the corresponding context (Casper & Krausch, 1981; Hacker et al., 2003; NLWKN, 2011; Voightländer & Mohr, 2008). Whether they are genotypically adapted to specific environmental conditions or a result of phenotypic plasticity is thus still unknown. T. Herden, M. Bönisch, & N. Friesen (unpublished data) analyzed 27 populations of *H. repens* in Germany with SSRs and found only low levels of variation within the analysed markers. There we found no genetically based separation into a *Terr* or *Aqu* cluster, suggesting differences due to phenotypic plasticity. However, our sample set was not aimed to address the taxonomic status of both forms. The ecotype hypothesis cannot be excluded based only on these results. Markers may fail to detect quantitative variation for adaptively important traits (Bekessy, Ennos, Burgman, Newton, & Ades, 2003; McKay & Latta, 2002).

If both forms appear to be ecotypes, it can have consequences on the conservation management. Ex situ conservation management for aquatic forms needs to be adapted as well as conservation at the natural sites. Additionally, this information might be interesting for plant breeders as both ecotypes may harbor specific traits of interest.

This comparison study aimed to answer the question of the taxonomical status of both forms by using simple sequence repeats (SSR) and intersimple sequence repeats (ISSR) data on a balanced sample set.

Additionally, the adaptation of both forms to drought stress was studied by measuring the relative water content (RWC) of leaves,

**TABLE 1** Provenances of the analysis populations (modified after T. Herden, M. Bönisch, & N. Friesen (unpublished data))

Lab-ID	GE-Sell ID	State	District	Commune	Form
1R	MV-GC-20120912-1400	MV	Demmin	Meesiger	Terr
5R	MV-DS-20131029-1030	MV	Müritz	Alt Schwerin	Terr
8R	NRW-DB-20150818-1831	NW	Paderborn	Delbrück	Terr
9R	NI-OM-20150812-0955	NI	Diepholz	Hüde	Terr
10R	SH-TIV-20150902-0900/0910/0920	SH	Plön	Blekendorf	Terr
12R	Bbg-SE-20150723-1634	BB	Havelland	Seeblick	Terr
16R	BY-GAP_FARC-20151021-1004	BY	Garmisch-Partenkirchen	Farchant	Aqu
18R	BY-KEH_NIED-20150908-1005	BY	Kelheim	Langquaid	Terr
19R	BY-KF_KAUF-20150814-1012	BY	Kaufbeuren	Kaufbeuren	Aqu
20R	BY-LL_BISC-20160828-1022	BY	Landsberg am Lech	Dießen am Ammersee	Aqu
21R	BY-MB_TRAC-20150811-1002	BY	Miesbach	Fischbachau	Aqu
22R	BY-MB_TRIN_20150802-1003	BY	Miesbach	Kreuth	Aqu
24R	BY-MN_SALG-20150804-1019	BY	Unterallgäu	Salgen	Aqu
25R	BY-MUE_MARS-20150829-1027	BY	Mühldorf a. Inn	Maitenbeth	Aqu
27R	BY-TS_WINK-20151114-1001	BY	Traunstein	Übersee	Terr

Notes: GE-Sell ID = reference IDs used in the project GE-Sell, states = federal states of Germany (BB = Brandenburg, BY = Bavaria, MV = Mecklenburg-West Pomerania, NI = Lower Saxony, NW = North Rhine-Westphalia, SH = Schleswig Holstein, ST = Sachsen Anhalt), form = form of *H. repens* (Terr—terrestrial; Aqu—aquatic).

system water content, and water loss during drought stress conditions. The stomatal index (SI) was measured for different water treatment levels. A small scale experiment was set up, to determine whether *H. repens* is capable of self-fertilization.

## 2 | MATERIAL AND METHODS

### 2.1 | Genetic analysis

#### 2.1.1 | SSR analysis

SSR or microsatellites are short stretches of repeated short nucleotide motifs. These motifs typically consist of mono-, di-, and tri-nucleotides, but even longer ones can be found. The repetitions of the motifs are mainly <100 base pairs (bp) long and can be found in all genomes (Tautz, 1989). They can show side-specific length variation because of the occurrence of different numbers of repeat units (Morgante & Olivieri, 1993). Most of these length differences are caused by the slippage effect during replication and accumulate over time (Tautz & Schlötterer, 1994). Using the polymerase chain reaction (PCR), with specific primer pairs flanking a specific microsatellite, it is possible to amplify and measure the exact bp length of a microsatellite. SSR markers are considered to be a reliable system for diversity studies as they are codominant and multiallelic (Baldwin, Pither-Joyce, Wright, Chen, & McCallum, 2012; Fu, Kong, Yingxiong, & Cameron, 2005; Geethanjali, Anitha Rukmani, & Rajakumar, 2018; Park, Lee, & Kim, 2009; Yasodha et al., 2018). They are neutral markers and are thus usually not subjected to natural selection (Holderegger, Kamm, & Gugerli, 2006; Kimura, 1983).

The data from T. Herden, M. Bönisch, & N. Friesen (unpublished data) were evaluated to investigate genetic differences between *Aqu* (16R, 19R, 20R, 21R, 22R, 24R, and 25R) and *Terr* (1R, 5R, 8R, 9R, 10R, 12R, 18R, and 27R) populations (Table 1). Counts for allelic richness, fixation index (*F*-Index), inbreeding coefficient  $F_{is}$ , private alleles, rare alleles, single locus genotypes (SLG), multilocus genotypes (MLG), and numbers of alleles were taken from the data analysis of T. Herden, M. Bönisch, & N. Friesen (unpublished data) (Table S2).

#### 2.1.2 | ISSR analysis

Intersimple sequence repeats (ISSR) are regions between microsatellite loci. In a PCR, only one primer containing an SSR motif is used, which amplifies multiple fragments with various length (Reddy, Sarla, & Siddiq, 2002; Zietkiewicz, Rafalski, & Labuda, 1994). Only regions between adjacent, inversely oriented SSRs are thus amplified (Zietkiewicz et al., 1994). Usually, the PCR products are visualized on an agarose gel, and the banding pattern is transformed into a binary matrix. Every band is treated as a single trait. By analyzing the matrix, kinship relations can be computed. Polymorphism can be detected due to mismatches in the priming site (changes in the SSR where the primer binds) or differences in length of the amplified sequences (Zietkiewicz et al., 1994). This method has been widely used for decades in population genetic studies and studies to characterize genetic divergence among species (Andiego et al., 2019; Kumar, Mishra, Singh, & Sundaresan, 2014; Reddy et al., 2002; Schlotteröer, Amos, & Tautz, 1991; Zietkiewicz et al., 1994).

DNA isolates were taken from T. Herden, M. Bönisch, & N. Friesen (unpublished data). An agarose gel documentation with 47 lanes was

used. Three individuals from each population (eight *Terr*—1R, 5R, 8R, 9R, 10R, 12R, 18R, and 27R and seven *Aqu* populations—16R, 19R, 20R, 21R, 22R, 24R, and 25R) were chosen for further investigation (Table 1). The isolated DNA was used directly in a PCR with 10  $\mu$ l Biozym red HS Taq master mix (Biozym Scientific GmbH), 1  $\mu$ l of corresponding primer (Table S1), and 1  $\mu$ l DNA template in a final volume of 20  $\mu$ l. PCR products were checked on an agarose gel. The bands were scored independently as either present (1) or absent (0) and summarized in a matrix. Polymorphism information content (PIC) values were calculated using the formula described previously in Roldan-Ruiz, Dendauw, Bockstaele, & Depicker, 2000. A principal component analysis (PCA) was performed using the function *dudi.pca* from the R package *ade4* (Bougeard & Dray, 2018; Chessel, Dufour, & Thioulouse, 2004; Dray & Dufour, 2007; Dray, Dufour, & Chessel, 2007).

## 2.2 | Self-fertilization test

Plants from two populations that were currently available (nine individuals from 9R and nine from a population from Austria) were potted in trays. These were then isolated from potential pollinators using transparent plastic hoods with *Drosophila* impermeable mesh for airflow. One control from each population was potted outside of the isolation hoods. The isolated individuals were pollinated by hand with their pollen. At the end of their vegetation period, the seeds were collected. Seeds were drawn randomly for germination tests.

## 2.3 | Dry stress experiment

Stolons from 15 *Terr* plants (population 9R) were potted in 10  $\times$  10 cm pots (the stolon was approximately 5 cm long with two leaves). For substrate, 173 g of "Einheitserde Special" (Einheitserdewerke Werkverband e.V., Sinntal-Altengronau, Germany) was used. Plants were grown for three weeks in a greenhouse to ensure that they have rooted successfully. During that time, all pots stood in trays filled with water to ensure that they were watered to their maximum water capacity. They were treated with extra light using one unit of the *KIND LED L600 grow light* (Santa Rosa, CA), until the start of the experiment. All plants were weighted (system water content (SWC) = weight of soil, pot, and plant) just before they were put into a climatic chamber (maximum run time for every run: 20 days, day temperature: 33°C; night temperature: 22°C; light: 14 hr; dark: 10 hr; rel. humidity >80%). The pots were weighed daily during the runs. During the experiment, the pots were not watered. To ensure that all plants grew under the same condition, the pots' positions in the chamber rotated every day. If plants lost all their leaves due to wilting, they were taken out of the chamber and watered immediately to their maximum water capacity, to prevent the loss of study material.

All plants recovered during a recuperation period of three weeks in the same greenhouse conditions as mentioned above. The experiment was then repeated with the same plants to assess potential adaptation.

The same experiment was conducted with plants from *Aqu* populations (population 16R, 22R, 24R), which were grown in soil for a time period of one year. For that, five individuals were collected at three natural sites (with the maximum distance between each

sample) and cuttings were used in the experiment. For every plant, the results were statistically evaluated by one-way analysis of variance (ANOVA), using the software R (R Core Team, 2017).

At the beginning of each run, one leaf from every plant was used to measure the RWC. For that, the weight (*W*) of a freshly harvested leaf was measured and put in a 50-ml centrifuge tube with 5 ml of distilled water for rehydration. As Arndt, Irawan, and Sanders (2015) already indicated, rehydration by floating leads to erroneous RWC estimates. Therefore, the leaves were put petiole first in the distilled water, making sure that the water level did not reach the lowest pair of leaflets. They were rehydrated for three hours in darkness under room temperature conditions, and the turgid weight (*TW*) was measured afterward. All leaves were left in a dry chamber with 10% relative air humidity overnight and weighted afterward to measure the dry weight (*DW*). The RWC was calculated using the formula of Weatherley (1950). The measurement was also carried out with leaves that exhibit a complete loss of turgor pressure. The system water loss (*SWL*) was calculated ( $SWL = (1 - SWC_{end}/SWC_{start}) \times 100$ ).

Tests for significance were made with the *geom\_signif* function using the R package *ggplot2*, and plots were drawn using the function *ggplot* from the R package *ggplot2* (Wickham & Chang, 2018).

## 2.4 | Stomatal index

To estimate the SI, nail polish impressions from the epidermis were made (as described in Miller & Ashby, 1968) from plants cultivated ex situ in the Botanical Garden of Osnabrueck, Germany. Ten impressions from the upper surface were made from all leaflet pairs of a leaf, to test whether there are significant differences between each leaflet pair. The same was done for the lower leaf surface. Pictures of impressions were made using a transmitted light microscope under 400 $\times$  magnification. Stomata counts (*SC*) and epidermis cell counts (*EC*) were quantified (guard cells were treated as a part of the stomatal apparatus). The observed surface area was measured (*A*), and the stomatal density (*SCD*), as well as the epidermal cell density (*ECD*), was calculated. Three pictures were taken from every leaflet pair, and the quantifications of the *SC* and *EC* were averaged. The SI was calculated for every leaflet pair using the equation from Salisbury (1928).

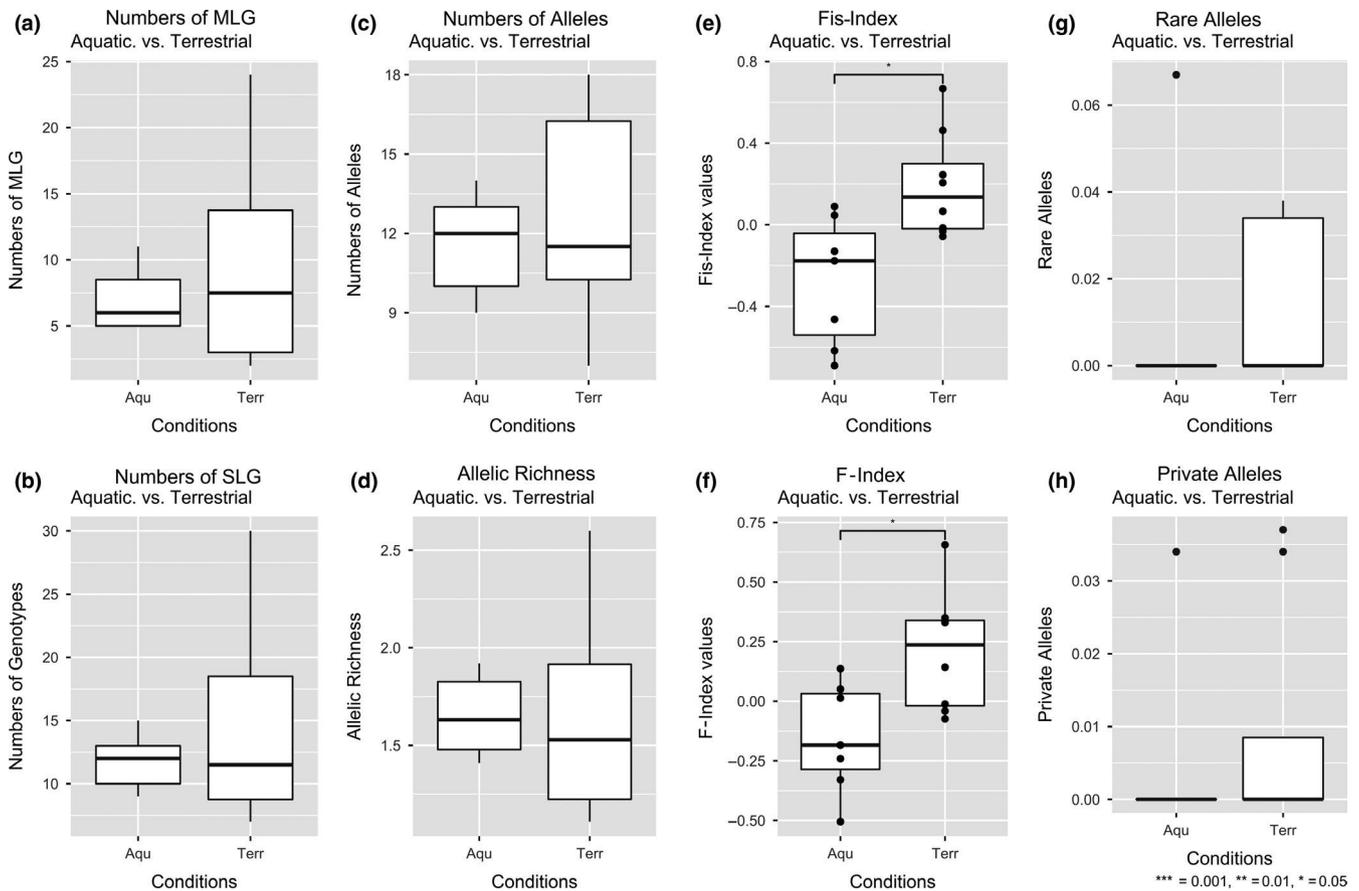
The SI was calculated for two different water treatment levels for every form: *Terr*—terrestrial form growing in pots with drainage with local weather conditions, *T-Wet*—terrestrial form watered to their maximum water capacity, *Aqu*—aquatic form growing under aquatic conditions, and *A-T*—aquatic form potted in soil and growing under the same conditions as *T-Wet*.

# 3 | RESULTS

## 3.1 | Genetic analysis

### 3.1.1 | SSR analysis

There were no significant differences in the numbers of MLG, SLG, alleles, allelic richness, rare alleles, and private alleles between *Terr* and *Aqu* plants (Figure 2a–d,g,h). As T. Herden, M. Bönisch, & N.



**FIGURE 2** Comparison between aquatic and terrestrial populations of *Helosciadium repens* in Germany using diversity parameters from the SSR analysis. (a) Numbers of multilocus genotypes, (b) numbers of genotypes, (c) numbers of alleles, (d) allelic richness, (e) inbreeding coefficient  $F_{is}$ -Index, (f) fixation index  $F$ , (g) counts of rare alleles, and (h) counts of private alleles. Asterisks are indicating significance levels

Friesen (unpublished data) showed, there is no genetically based separation into a *Terr* or *Aqu* cluster. However, there were significant differences ( $p < .01$ ) in the  $F$ - and  $F_{is}$ -Indices between both forms. The  $F$ -Index values for the *Aqu* populations were mostly negative indicating an excess of heterozygosity. There were only three populations (20R, 22R, and 16R) with positive values. Most of the *Terr* populations had positive  $F$ -Index values indicating an excess of homozygosity. Only five populations (2R, 10R, 11R, 12R, and 18R) showed negative values (Figure 2e). The same was true for  $F_{is}$ -Index values (Figure 2f). Only two *Aqu* populations had positive values (20R and 22R) and one exhibited a  $F_{is}$ -Index value of zero (23R). Five *Terr* populations (3R, 10R, 11R, 12R, and 18R) had negative values (Table S2). The rest of the *Terr* populations exhibited positive  $F_{is}$ -Index values.

### 3.1.2 | ISSR analysis

Only eight out of 26 tested ISSR markers produced evaluable polymorphic bands. A total of 108 bands were amplified out of which 64 were polymorphic, and 42 were monomorphic bands (Table S1). The percentage of polymorphic bands ( $P\%$ ) per primer ranged from 77.8% in UBC813 to 38.9% in UBC834. The average percentage of

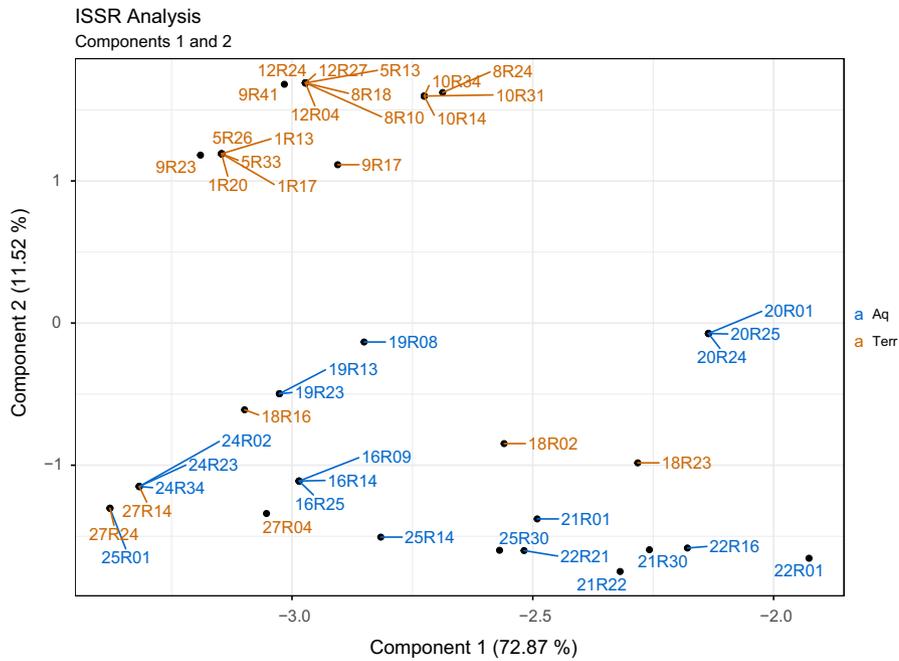
the polymorphic band was 60.7%. PIC values spanned from 0.4409 in UNC810 to 0.2647 in HB15 (Table S1).

The first three components of the PCA explained 87.87% of the data (comp. 1:72.87%, comp. 2:11.52%, and comp. 3:2.67%) (Figure 3). Two distinct clusters were visible. One was composed of Bavarian populations and one of the populations from northern Germany. This partitioning coincides with the SSR analysis of T. Herden, M. Bönisch, & N. Friesen (unpublished data). Separation into *Terr* or *Aqu* clusters was not observed.

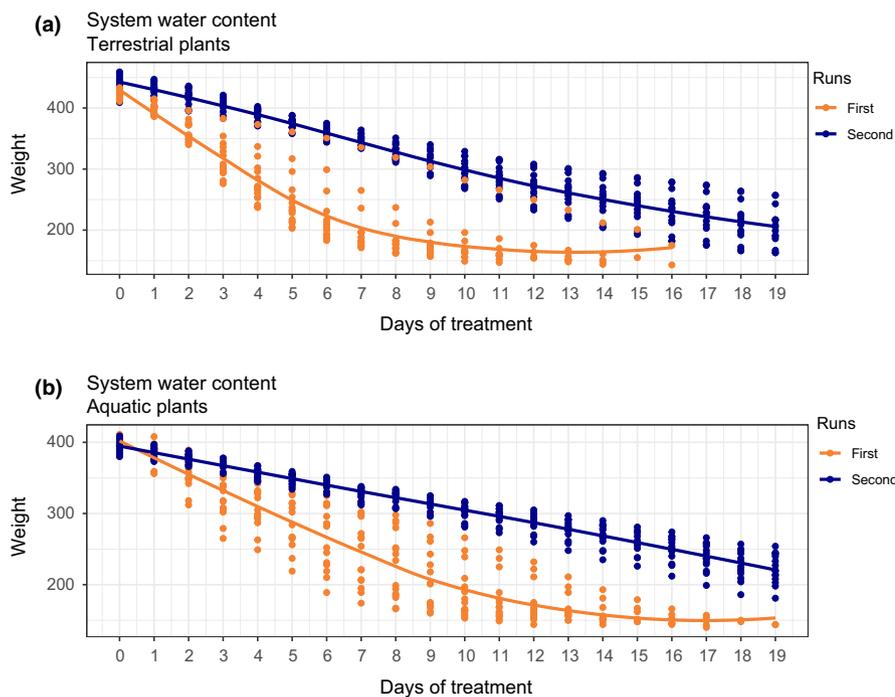
Both analyses (SSR and ISSR) showed congruent results, namely a split between Northern and Southern populations (Figure 3) (T. Herden, M. Bönisch, & N. Friesen, unpublished data).

### 3.2 | Self-fertilization test

There was an evident difference in the number of seeds between the isolated and their control pots. However, due to high humidity in the isolated trays, some of the inflorescences and infructescences started to rot. Therefore, a test for statistical significance was not possible. Nevertheless, the isolated plants produced seeds when fertilized with their pollen. Randomly selected seeds were able to germinate.



**FIGURE 3** Principal component analysis of the ISSR data of eight terrestrial and seven aquatic populations. Blue = Aq=aquatic population, orange = Terr=terrestrial populations; Lab IDs = first digits including the letter R (see Table 1); individuals = digits after the letter R



**FIGURE 4** Daily system water content of the first and second runs during the drought stress experiment. (a) Terrestrial plants, (b) potted aquatic plants; orange lines represent smoothed conditional means of the first runs, and blue lines represent smoothed conditional means of the second run

### 3.3 | Dry stress experiment

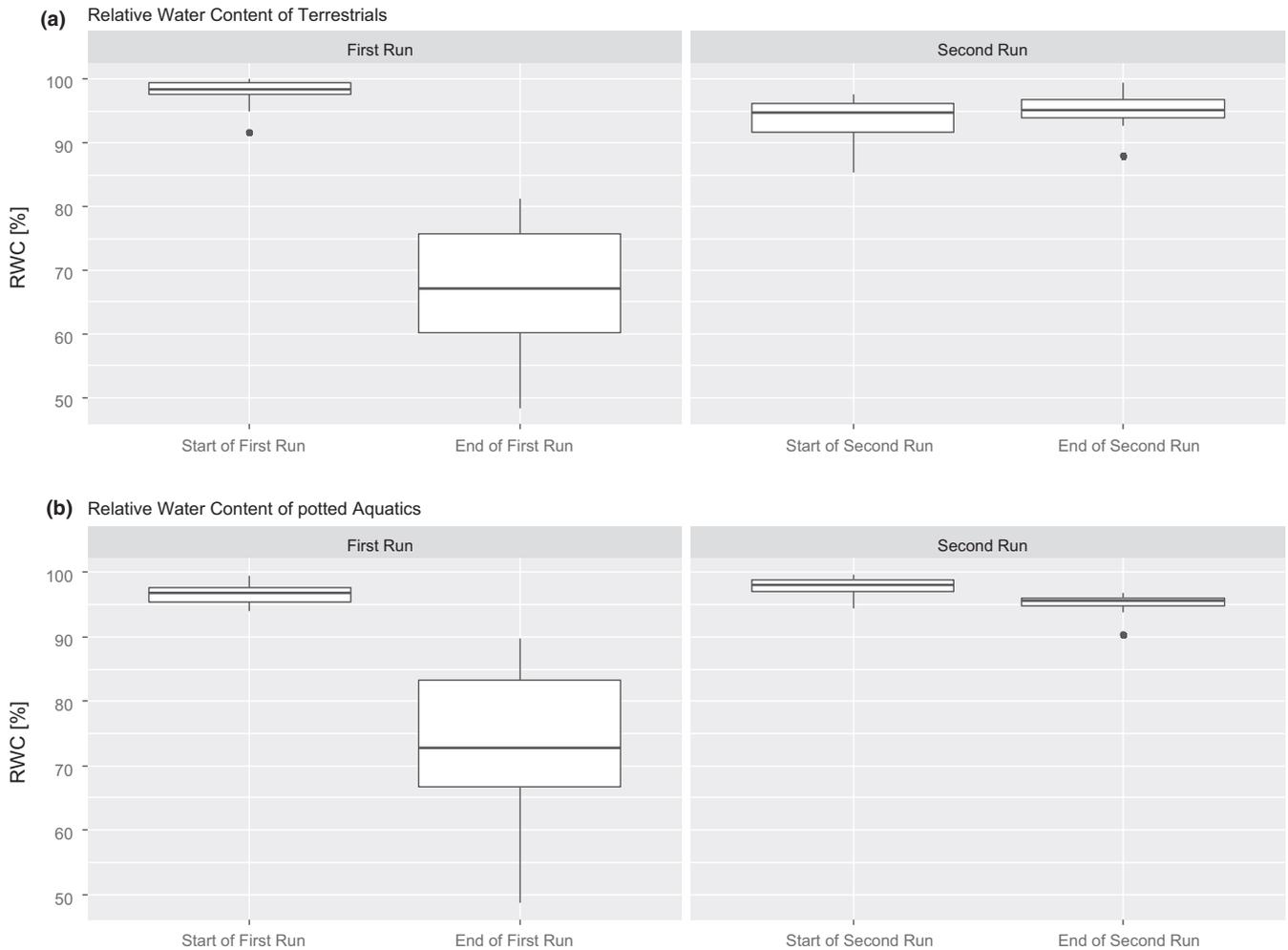
#### 3.3.1 | Terrestrial plants

During the first run of the *Terr* plants, none of the plants endured the scheduled time of 20 days without a complete loss of leaves. About 46% of the plants dropped all leaves during the first nine to 11 days. Two plants shed all its leaves after 16 days (Figure 4a). On average, there was a SWL of 64% at which plants lost all their leaves. After 16 days, the control pot had an SWL of 51%. The relationship

of the variables was explained best with a polynomial regression ( $0.9902 < \text{adjusted } R^2 < 0.9993$ , median: 0.998) instead of a linear regression ( $0.8663 < \text{adjusted } R^2 < 0.9917$ , median: 0.9167) (adjusted  $R^2$ , slopes ( $b$ ), intercept ( $a$ ), and residual standard deviation (res. SD) are given in Figure S1a,b). Only plant VI had an even water loss which was comparable to the linear regression (Figure S1a).

The new leaves that grew back during the recovery period were smaller and stiffer.

In the second run, all plants endured the scheduled time of 20 days without a complete loss of leaves (Figure 4a). The plants



**FIGURE 5** Relative water content at the start and the end of the first and second runs during the drought stress experiment. (a) Terrestrials, (b) potted aquatics

showed signs of withering, after an average SWL of 55% (39%–65%). The control pot had an SWL of 38%. Overall, the SWC was significantly higher during the second run. The adjusted  $R^2$ s were higher than 0.98, except for plants II, IV, and V ( $>0.97$ ) (Figure S1a). The relationship of the variables during the second run almost fits a linear regression in all investigated plants (Figure S1a,b). The slopes of the linear regressions were between  $-16.6$  and  $-8.1$  (median:  $-12.76$ ).

Figure 4a shows the smoothed conditional means of all plants during the first and second runs. The curve is, except for the slope ( $b_{\text{control}} = -7.9948$ ,  $b_{\text{median}} = -12.7554$ ), comparable to the one from the control pot (Figure S1b plant control).

The RWCs of leaves at the start of run one (with full turgor pressure) and the end of the run one (complete loss of turgor pressure) were significantly different (Figure 5a). The leaves lost on average 31.44% (lowest: 15.1%; highest: 50.49%) of water. In run two, the RWCs did not differ significantly between the beginning and the end of the run (Figure 5a). The leaves had a negative water loss and gained on average 1.76% (lowest:  $-13.75\%$ ; highest: 3.75%) of water.

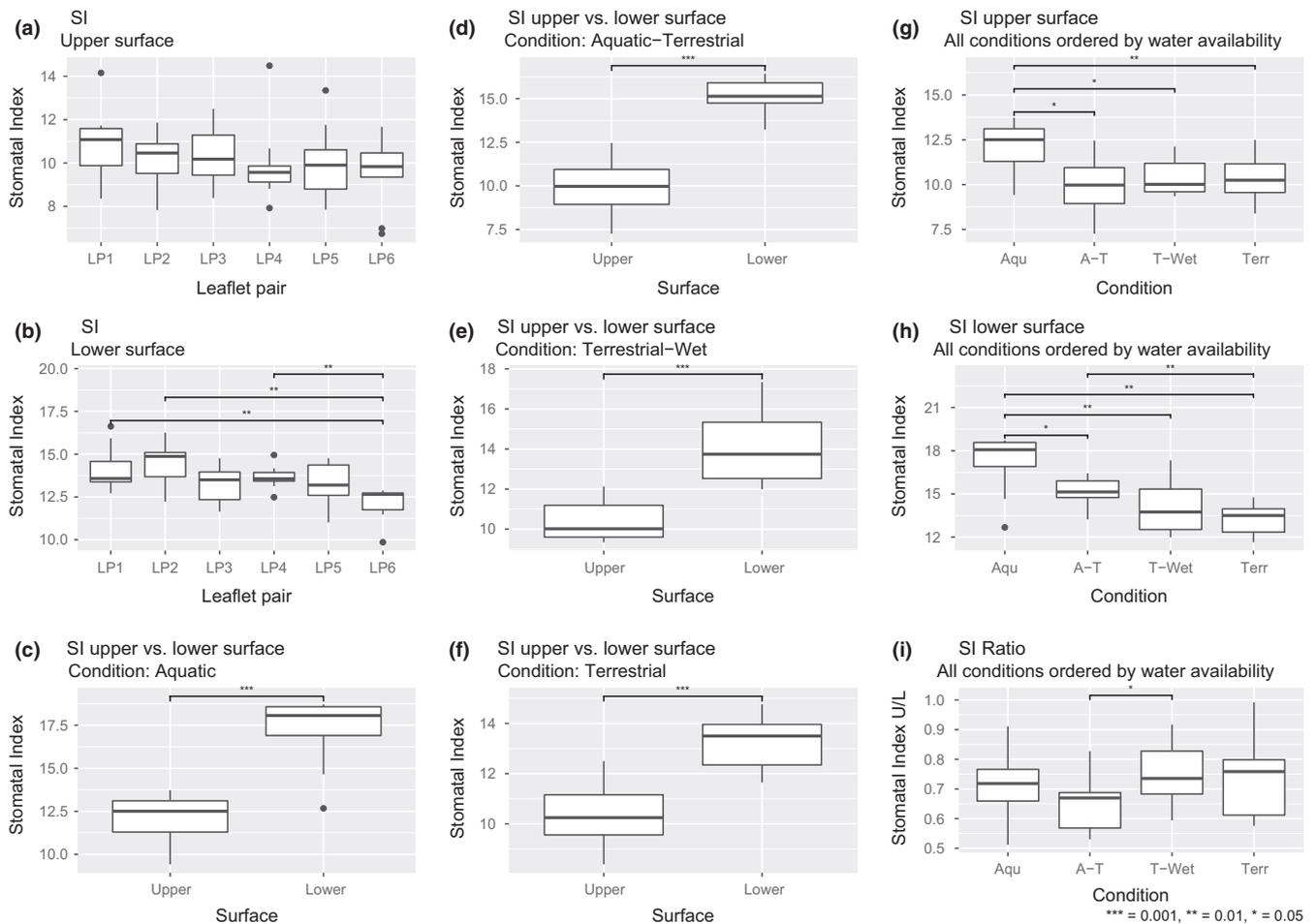
### 3.3.2 | Potted aquatics plants

During the first run, only one plant out of 15 endured the scheduled time of 20 days without a complete loss of leaves (Figure 4b). At day 15, 53% of the plants lost all their leaves. The average SWL was 63% and ranged from 61% to 65%. The control pot had an SWL of 46%.

The adjusted  $R^2$  for the linear regression for the SWC curves of each plant was between 0.9929 and 0.8864 (median: 0.9549) with a res. SD between 28.49 and 7.279 (median: 17.61). The curves of plant III, VIII, and X are very close to that of the linear regression with the adjusted  $R^2 > 0.98$  and the res. SD  $< 9.2$  (Figure S1c,d). However, the relationship of the variables was best explained with polynomial regression (adj.  $R^2$ : 0.9893–0.9967, median: 0.9945; res. SD: 9.092–4.957, median: 6.479) (Figure S1c,d).

The new leaves that grew back during the recovery period were smaller and stiffer.

In the second run, all plants endured the scheduled time of 20 days without a complete loss of leaves (Figure 4b). The plants showed signs of withering at an average SWL of 45% (36%–57%). The control pot had an SWL of 35%. The adj.  $R^2$  was between 0.9968



**FIGURE 6** Comparison of the stomatal index (SI). (a) SI from the upper surfaces of different leaflet pairs, (b) SI from the lower surfaces of different leaflet pairs, (c) SI comparison of the upper and lower surface of aquatic plants, (d) SI comparison of the upper and lower surface of potted aquatic plants, (e) SI comparison of the upper and lower surface of terrestrial plants grown in wet conditions, (f) SI comparison of the upper and lower surface of aquatic plants, (g) SI comparison of the upper surface of all conditions, (h) SI comparison of the lower surface of all conditions, (i) comparison of the SI ratio between the upper and lower surfaces of all conditions. Asterisks are indicating significance levels

and 0.9991 with a res. *SD* between 3.464 and 1.528. The curves fit the linear regression (Figure S1c,d). For plants III and VIII, the curves fit the linear regression best in the second run (Figure S1c). The slopes of the linear regressions were between  $-11.92$  and  $-7.13$  (median:  $-9.08$ ). The slope of the linear regression of the control pot was  $-6.62$  (Figure S1d plant control).

Figure 4b shows the smoothed conditional means of all plants during the first and second runs. The curve is, except for the slope ( $b_{\text{control}} = -6.62$ ,  $b_{\text{median}} = -9.08$ ), comparable to the one from the control (Figure S1d plant control).

The RWCs between leaves at the start of the run one (with full turgor pressure) and those at the end of the run one (complete loss of turgor pressure) differed significantly (Figure 5b). The leaves lost on average 23.95% (lowest: 7.33%; highest: 49%) of water. In run two, the RWCs were again significantly different when comparing the beginning and the end of the run. The leaves lost on average 2.51% (lowest:  $-0.3\%$ ; highest: 6.1%) of water. The difference in water loss between both runs was significant ( $p < .001$ , data not shown).

The RWC at the start of both runs was significantly different, comparing both conditions (*Aqu* and *Terr*). On average, the differences were 1.28% in the first run and 4.3% in the second run. At the end of both runs, the RWCs in both conditions were not significantly different anymore ( $p < .001$ , data not shown).

### 3.4 | Stomatal index

There were no significant differences between the different leaflet pairs in a leaf (Figure 6a,b). In all conditions (*Aqu*, *A-T*, *T-Wet*, and *Terr*), the SI of the upper surface was significantly lower than the SI from the lower surface ( $p < .001$ ) (Figure 6c-f). On the upper surfaces, the SI was significantly higher for *Aqu* than all other conditions with different levels of significance (Figure 6g). There were no significant differences between conditions *A-T*, *T-Wet*, and *Terr*.

On the lower surfaces, the SI of *Aqu* was significantly higher (with different levels of significance) in comparison with the SI of plants grown under other conditions (Figure 6h). There was a significant

difference between the SI of *Aqu* and *Terr*. However, there was no significant difference between both forms grown under the same condition (*A-T* and *T-Wet*).

The ratio (SI upper/SI lower) between the upper and lower surfaces for each condition was analyzed (Figure 6i). The only significant difference was detected between *A-T* and *T-Wet* ( $.01 < p < .05$ ).

### 3.5 | General observations

Five cuttings from every *Aqu* populations were potted and the rest grown in small trays with water. All plants, in the trays with water and the pots, build inflorescences and infructescences.

## 4 | DISCUSSION

Two main results derived from this study: (a) The analyses of the SSR and ISSR data showed similar outcomes and no significant separation into ecotypes; (b) the differences in morphological characters of the two forms faded when plants were grown under the same conditions.

### 4.1 | Genetic comparison

Both fingerprinting methods (SSR and ISSR) together portray the genetic diversity of the entire genomes of all investigated individuals. Nevertheless, most populations can be genetically told apart from each other; both forms are not genetically differentiated (Figures 2 and 3). Therefore, a taxonomical division based on molecular data is not justified.

The only significant difference recovered from the genetic data was from the  $F$ -statistics (Figure 2e,f; Table S2). The heterozygote excess, revealed by a negative  $F_{is}$ , can be caused by asexual propagation (Stoekel et al., 2006). Four out of the seven *Aqu* populations exhibited negative  $F$  and  $F_{is}$  values. These findings confirm the observations that these populations tend to grow clonally (Casper & Krausch, 1981; Schossau 2000 cited in Hacker et al., 2003; NLWKN, 2011). However, three of them have positive  $F$ -statistic values. A heterozygote deficiency (homozygote excess) is revealed by positive  $F_{is}$  values and can be caused by self-fertilization. This is mainly the case in the *Terr* populations.

In *Aqu* populations, most of the leaves are partially submerged due to floating (pers. observation). When leaves are submerged, they encounter an oxygen shortage (Mommer & Visser, 2005). Hypoxia triggers the ethylene production and thus the adjustments to the submerged conditions such as development of aerenchyma (Drew, Jackson, Giffard, & Campbell, 1981; Gunawardena, Pearce, Jackson, Hawes, & Evans, 2001; Jackson & Armstrong, 1999; Jackson, Fenning, Drew, & Saker, 1985; Kordyum, Kozeko, Ovcharenko, & Brykov, 2017; Yamauchi, Shimamura, Nakazono, & Mochizuki, 2013) or submergence-acclimated leaf forms (Kuwabara, Ikegami, Koshihara, & Nagata, 2003; Kuwabara, Tsukaya, & Nagata, 2001). In the case of *H. repens*, it possibly inhibits the flowering as it does in *Ipomoea*

*nil* (L.) Roth (Suge, 1972; Wilmowicz, Kęsy, & Kopcewicz, 2008) or in *Xanthium pungens* Wallr. (Abeles, 1967). The plants in the tray were in contact with the bottom and were thus able to sustain upright leaves above the water surface. Due to fluctuations in the water level at the natural sites, the very similar conditions can occur possibly leading to infrequent flowering. Burmeier and Jensen (2008) observed that seeds were able to germinate even under water. Therefore, seed recruitment during low water seems possible and could explain the positive  $F_{is}$  values.

However, these interpretations remain largely hypothetical and constitute a basis for further research.

### 4.2 | Morphological comparison

#### 4.2.1 | Drought stress

Both forms undoubtedly adapted between the first and the second runs (Figure 4). This adaptation is also visible in the RWC values of the leaves (Figure 5). During the second run, the RWC values of the leaves did not drop as much as in the first runs (Figure 5). In some cases, the leaves even gained water and plants grew new leaves during the run (pers. observation).

#### 4.2.2 | Stomatal index

There were no significant differences between forms (Figure 6). One could interpret the differences in the SI of the upper surface of all conditions as a plastic reduction in SI caused by reduced water availability (Figure 6h). The difference in the ratio of upper and lower surface SI between *A-T* and *T-Wet* was likely due to the variation in the data and would probably disappear if more repetitions were carried out (Figure 6i). Had this been a genotypic trait, both extreme conditions (*Aqu* and *Terr*) would have shown differences in the SI.

## 5 | CONCLUSION

In general, neither molecular data nor the results from water-manipulating experiments alone can rule out the hypothesis of ecotypes. Molecular markers may fail to detect differences (Bekessy et al., 2003), and there could be other ecological factors in which the two forms behave differently. Billet, Genitoni, and Bozec (2018) analyzed aquatic and terrestrial morphotypes of *Ludwigia grandiflora* (Michx.) Greuter & Burdet and based on morphological traits they found that the terrestrial morphotype outcompetes the aquatic one. However, they did not perform molecular analyses; thus, the molecular basis of *L. grandiflora* adaptation remains unknown.

Ecotype hypotheses can be addressed only when morphology as well as genetic foundation studies is combined (McKay & Latta, 2002). In a study on *Alternanthera philoxeroides* (Mart.) Griseb., Geng et al. (2007) used molecular data (ISSR) and common garden

experiments to test the ecotypes hypotheses for aquatic and terrestrial forms. Their data supported, however, the plasticity hypothesis. For *Coccothrinax argentata* (Jacq.) L.H.Bailey, Davis, Lewis, Francisco-Ortega, and Zona (2007) found minute differences in the ISSR analysis between the mainland and insular populations. However, they found a great deal of plasticity in the traits included in the study that do not support a separation into different taxa. In *Ageratina adenophora* (Spreng.) R.M.King & H.Rob., the authors found evidence for phenotypic plasticity after checking 16 populations with ISSR and common garden experiments (Zhao, Yang, & Xi, 2012). Noel, Machon, and Porcher (2007) analyzed *Ranunculus nodiflorus* L. populations in France with microsatellites and common garden experiments. They found no genetic diversity and strong evidence favoring phenotypic plasticity.

Since our molecular data provide strong evidence against the ecotype hypothesis and the morphological differences disappeared during a simple drought stress experiment, the results can only lead to one explanation: phenotypic plasticity. Moreover, the drought stress experiment showed that plants that experienced drought stress performed better when subjected to drought stress again. This adaptive plasticity in this species enables it to endure short periods of drought stress and periods of water stress (Longa, 2019). It also gives the plants an advantage over competitors in zones of water fluctuations such as wet pastures and littoral zones, where this species naturally occurs. The ability of self-fertilization may benefit *H. repens* in environments where pollinators are scarce.

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

T.H. and N.F. conceived the ideas and designed methodology; N.F. supervised the study; T.H. did the laboratory work, conducted the experiments, and collected and analyzed the data; T.H. led the writing of the manuscript. Both authors contributed critically to the draft. The authors have no conflicts of interest to declare.

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## OPEN DATA BADGE



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://doi.org/10.5061/dryad.m0cfxpnzg>.

## DATA AVAILABILITY STATEMENT

Data is available under <https://doi.org/10.5061/dryad.m0cfxpnzg>.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## **Chapter 5**

### GENERAL DISCUSSION AND CRITICAL POINTS



## The Concept

The model of the genetic reserve described by Hawkes et al. (1997) was not always practical and was thus needed to be adjusted. In cases of small sites surrounded by agricultural fields, the transition, buffer and core zones would have shared the same borders. Additionally, the decision on the zone borders highly depends on the borders of the property of the involved participants. In the concept described by Hawkes et al. (1997), the borders represent mostly the demands of the populations, and the potential political barriers are subordinated. Therefore, the model had to be altered (Frese et al. 2018, Chapter 2).

In the first step, *the provision of distribution data* (Chapter 2), the data excerpt from the federal agencies were very heterogeneous. Scientific studies that have a Germany-wide focus would benefit (concerning time and effort) if systems were based on national standards. This criticism refers mainly to the coordinate reference system, taxonomic systems and measurement systems. Every federal state uses their local Gauß-Krueger projection, and the geo-information systems (GIS) are not used in all federal states (own observation). Taxonomic status should be up-to-date, and a standardised measurement for abiotic and biotic factors such as population size is desirable. However, some efforts have been already made in terms of descriptors for PGRDEU (Sensen 2015), FloraWeb (BfN, 2018b) or Deutschlandflora (BfN, 2019).

As mentioned in Chapter 3, the decision for the MAWPs cannot be based entirely on scientific reasoning. Social aspects are an essential factor. The communication from early on with all the involved parties led to active cooperation and information exchange. Information that would otherwise be hard to come by or expensive. The best medium was personal communication (personal communication Dr Lothar Frese and Maria Bönisch, JKI Quedlinburg).

In Table 1 (Chapter 2), the column *preservation status of the federal states*, the entries for *H. repens* were mistakenly copied from *A. graveolens*. It should read BW: 1, BY: 2, BE: 0, BB: 2, HH: 0, HE: 0, MV: 2, NI: 1, NW: 1, RP: 0, SL: 0, SN: nv, ST: 1, SH: 1, TH: nv. Nevertheless, we were unable to identify any *H. repens* populations in Baden-Württemberg.

Until now, the eight steps process described in Chapter 2 for establishing genetic reserves turned out to be successful. The last two steps, (7) *the planning and the establishment*, and (8) *operation of genetic reserve sites*, are the most crucial, as they include cooperation agreements and negotiations with all involved parties.

Since June 4<sup>th</sup> 2019, the first two genetic reserves for *H. repens* in Europe were successfully established on the peninsula Großer Schwerin in Mecklenburg-Western Pomerania (3R), and the population at the Hohennauener See in Brandenburg (12R).

## Genetics

It could be argued that testing the 49 SSR markers (Chapter 3) with more than three populations would have revealed more useful markers (unknown reviewer). From the author's point of view,

this argument is unsubstantial. When using DifferInt, missing data points lead to the exclusion of an individual. Therefore, only markers that recovered bands in all the investigated populations were used. Thus, the eleven originally picked SSR markers from the test were reduced to six, to include all 27 populations. Had the test of the 49 markers been done on all populations, it would have resulted in the same outcome.

Six SSR markers might seem to be insufficient to analyse the whole genome diversity (unknown reviewer). However, Abbasov et al. (2019) investigated the genetic diversity of six *Aegilops* L. species with only five SSR markers and were able to distinguish all six species by genetic clustering. Furthermore, El Zerey-Belaskri et al. (2018) analysed *Pistacia atlantica* accessions with six SSR markers and were able to distinguish four genetic groups. González-Díaz et al. (2018) analysed *Pinus sylvestris* with six nuclear genome SSRs successfully. Mangini et al. (2010) stated that they were able to distinguish all genotypes of durum wheat cultivars with only two SSR marker. Regardless, in chapter 4, a subset of the populations was analysed with eight ISSR markers that identified similar genetic clusters as did the SSR analysis. The observed diversity of the species in Germany was very low (six out of 49 SSR and eight out of 26 ISSR tested produced analysable PCR products).

In chapter 3, the DAPC did not recognise clustering according to the eco-geographic units (EGU). However, we cannot exclude that a possible adaptation to EGUs did happen. SSRs are namely neutral markers and are thus usually not subjected to natural selection (Kimura 1983; Holderegger et al. 2006).

It should be stated that the genetic differences revealed in the SSR analysis were only snapshots of a current state in space and time and thus not represent the actual dynamic diversification. To further approve the choice of 22R and 18R, multiple sampling and further analyses would be desirable.

## **MAWPs**

The populations which were chosen based on scientific reasoning were 18R and 22R. However, 22R is an aquatic population. As the availability of PGR (as germplasm) is an important feature of genetic reserves, one could have argued that choosing 22R as a MAWPs is of a disadvantage. However, in chapter 4, we found out that aquatic populations can indeed produce flowers. To secure seeds from such populations, one could cultivate sampled plants in botanical gardens, where the capture of seeds is less complicated. With this method, the obtained genetic diversity will only represent a fraction of the genetic diversity of the natural population. Thus, careful consideration of the sampled individuals and multiple repeats with newly sampled plants would be necessary.

## **Strategies for management**

There are different strategies on how to manage reserve sites, and in this thesis, a detailed description of two of them is provided. In biotope or biocenosis conservation, the focus is on the preservation of the diversity of habitats and their entire biocenosis (Jedicke 2001). Sturm (1993) suggested the protection of natural processes, which is based mainly on non-intervention. Jedicke

(2001) distinguishes two forms. The segregated one, where natural dynamics are conserved, and succession can take place and the integrative one, which is focused on cultural landscapes and the protection of the anthropogenic processes that led to these landscapes.

*Helosciadium repens* flourishes under extensive grazing management (McDonalds and Lambrick 2006; Burmeier and Jensen 2009) and disappears when natural succession takes place (Burmeier and Jensen 2008, 2009; Naturschutzring Dümmer E.V. 2015). Therefore, segregated process conservation methods seem highly inappropriate. A careful reconsideration of maintenance procedures, such as creating disturbances in the vegetation, crazing (Rosenthal and Lederbogen 2008; Burmeier and Jensen 2009), reduction of bushes and woody plants (own observation), reintroduction of material from conservation cultures and water regulation (Burmeier and Jensen 2009; Naturschutzring Dümmer E.V. 2014, 2015) is needed to successfully conserve the species at the sites. For that, periodical monitoring is essential.

### **Phenotypic plasticity**

Both forms are genetically identical concerning the observed markers. The internal transcribed spacer region (ITS1+5.8S+ITS2) of both forms was additionally amplified and resulted in identical sequences (data not shown). As the external transcribed spacer (ETS) usually exhibits a higher diversity (Baldwin and Markos 1998), there is a possibility, albeit small, that the ETS would show differences.

Nevertheless, all analyses suggest that both forms belong to the same taxon level. Additionally, the dry stress experiment showed, that all morphological features that implied a differentiation of the forms are dependent on environmental conditions and disappear when growing both forms under the same conditions.



## **Chapter 6**

### CONCLUSION



The genetic analyses showed that *H. repens* had a low level of genetic diversity and the populations a low level of differentiation. This was also visible in the DAPC. The clustering that revealed groups with predominantly north or predominantly south distribution was, however, transregional in these predominant areas. A more delicate division such as EGUs could not be detected.

The non-random mating and the low genetic variation is most likely a result of self-fertilisation or preferential mating. No migrations between the population were observed.

The northern populations had a lower genetic diversity in comparison with the southern populations. The influence of the last glacial maximum and the glacial European history can easily explain this discrepancy. However, only a broader study on a scale of the whole distribution area would answer this hypothesis. It could point out the refuge areas of this species, during the unfavourable glacial conditions.

For the conservation in Germany, we proposed 14 MAWP and two of them are currently already in existence.

ISSR and SSR, as well as ITS sequences, were not able to discriminate between both forms of *H. repens* recognised in the literature. The ISSR analysis confirmed the results of the SSR analysis. The North-South discrepancy was visible in the PCA of the ISSR data. Aquatic forms turned out to be as much genetically diverse as terrestrial forms.

Both forms reacted equally to the same environmental conditions. There were no significant differences, either in the stomatal index or in the reaction and subsequent adaptation to the drought stress conditions. Aquatic populations started to flower after only the water level was lowered to a point, where the leaves were no longer submerged. The role of ethylene is hypothesised in the inhibition of flowering. All observed differences are caused by phenotypic plasticity. A separation in taxon level is therefore not justified.

This study represents the next fundamental step towards the conservation of the CWR. Centuries of studies and discussions have led to the first genetic reserve in Europe. With this study, a blueprint was laid out that waits for the next CWR taxon to be processed. However, the continuation in research implies that further fundings will be granted, and demonstration projects will be transferred into long-term missions. Studies like these may be expensive, but the benefits we and all the future generations might gain are by far outweighing the costs.

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**Botanischer Garten, Osnabrück**

Wissenschaftliche Hilfskraft

- verantwortlich für die Sammlung des Index Seminum
- unterstützender Sammler für die Loki Schmidt Genbank für Wildpflanzen

## Ausbildung

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04/2015 – 10/2019

**Universität Osnabrück**

Promotion

“Genetic analysis of *Helosciadium repens* (Jacq.) W.D.J.Koch populations in Germany – Fundamental research for conservation management.”

10/2009 – 09/2012

**Universität Osnabrück**

Masterstudiengang: Biologie der Organismen

Abschluss: Master of Science (Note: 1,5)

“Herkunft, Taxonomie und Biogeographie der Sektion *Schoenoprasum* (Gattung *Allium*)”

10/2006 – 09/2006

**Universität Osnabrück**

Bachelorstudiengang: Biologie der Organismen

Abschluss: Bachelor of Science (Note: 2,3)

“Molekulargenetische Populationsanalysen von *Allium ursinum* L. in Süd Niedersachsen und NRW”

02/2003 – 12/2005

**Comenius Kolleg, Mettingen**

Abschluss: Abitur (Note 1,8)

2001 – 2002

Unterbrechung des Ausbildungsweges durch Zivildienst

1999 – 2001

**Creditreform Unger KG, Osnabrück**

Abschluss: Bürokaufmann

1998 – 1999

**Berufsbildende Schule, Melle**

Höhere Handelsschule Wirtschaft

Abschluss: Erweiterter Realschulabschluss

1996 – 1998

**Gymnasium, Melle**

Abschluss: Mittlere Reife

## Bibliography

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