Ecology and Hybridization Potential of Two Sympatric Metallophytes, the Narrow Endemic *Crepidorhopalon perennis* (Linderniaceae) and its More Widespread Congener *C. tenuis*

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ABSTRACT

Crepidorhopalon perennis is a metallophyte critically endangered by mining activities and currently known from only one site on copper-rich soils in Katanga (Dem. Rep. Congo). It is closely related to the annual *C. tenuis*, also a rare metallophyte, but with a broader geographical range. We investigated the variation in morphometric traits and ecological niches (based on edaphic conditions and pollinator assemblages) of *C. perennis* and *C. tenuis*, to evaluate the risk of inter-specific competition, and their potential for hybridization to ascertain if *C. perennis* might be at risk of genetic swamping by its more widespread congener. We examined whether species were found under sympatric or parapatric settings with opportunity for hybridization (or gene exchange). Such knowledge is essential for implementing restoration management protocols, including the introduction of *C. perennis* into substitution sites where *C. tenuis* might be already present. Fourteen morphological characters and 11 soil variables were measured and visiting pollinator species were identified at the site where the two species co-occur. Our results show that the two species can be distinguished based on their morphological traits, show niche overlap based on edaphic properties, and share the same pollinator assemblage. In addition, no morphologically intermediate individuals could be detected, suggesting no hybridization, and that the two species may be reproductively isolated. We conclude that *C. perennis* conservation and restoration operations can be realized in substitution sites where *C. tenuis* may be present, with the need, however, to evaluate the potential effect of sharing a pollinator assemblage on reproductive success of both species.

Key words: edaphic endemism; *ex situ* conservation; geobotany; Katanga (Dem. Rep. Congo); metallophyte; plant-pollinator interactions; plant-soil relations; rarity.

MANY EDAPHICALLY ENDEMIC PLANTS, WITH RESTRICTED DISTRIBUTIONS AND SPECIFIC HABITAT requirements, are declining or have already gone extinct as a result of habitat destruction, fragmentation and degradation (*e.g.*, Woodruff 2001, Jacobi *et al.* 2007). In addition to strongly decreasing population sizes, habitat fragmentation and degradation may increase the risk of hybridization between closely related species by bringing formerly allopatric species into sympatric settings (*e.g.*, Rhymer & Simberloff 1996, Lamont *et al.* 2003, Parsons & Hermanutz 2006).

Hybridization, especially when occurring between a rare plant species and a more common congener, may lead to a genetic assimilation of the rare taxon by the common one through genetic swamping, leading to the loss of its unique characteristics, and ultimately to its extinction (*e.g.,* Rieseberg 1991, Levin *et al.* 1996, Rhymer & Simberloff 1996, Lamont *et al.* 2003, Parsons & Hermanutz 2006). Reproductive-isolating mechanisms,

however, might prevent or limit between-species hybridization, such as differences in pollinator assemblages and flowering periods, incompatibility between pollen and stigmatic tissue, hybrid seed abortion, and reduced survival of the hybrid progeny (Rhymer & Simberloff 1996, Arnold 1997, Campbell *et al.* 2002, Pascarella 2007). Hybridization between two taxa can be detected by several methods (Wilson 1992, Rieseberg & Ellstrand 1993, McDade 1997, Parsons & Hermanutz 2006). The use of molecular markers gives reliable results (Hegarty & Hiscock 2005, Morgan-Richards *et al.* 2009), but their development may be time-consuming and costly. Because hybrids may consist of morphologically intermediate forms, morphometric trait analysis has also been largely used. This approach may be particularly useful when molecular markers are not available or difficult to develop (Soltis & Gitzendanner 1999, Will & Rubinoff 2004).

With increasing destruction of native habitat, *in situ* preservation for rare and endemic plant species is becoming difficult and other viable solutions for sustainable conservation need to be found. *Ex situ* conservation in botanical gardens (possibly

combined with reintroduction upon habitat restoration), and plant relocation in other wild sites sharing similar ecological conditions are often proposed (Whiting *et al.* 2004). These conservation practices, however, might accidentally lead to hybridization between the rare, threatened species and more widespread congeners that may already be present in the potential introduction sites or in the garden, threatening the genetic integrity of the taxon to be preserved (Maunder *et al.* 2004a). This new coexistence might also lead to competition for space, resources or pollinators if they occupy similar ecological niches (Brown *et al.* 2002, Bachmann *et al.* 2005). This might compromise the long-term persistence of the introduced taxon, especially if it is not possible to create large populations. It is therefore essential, before starting any introduction or *ex situ* conservation planning, to properly evaluate the potential risk for hybridization between the endemic and the closely related species that may already occur in the substitution sites. Additionally, the risk for competition also needs to be assessed by characterizing their ecological niches. To our knowledge, only a few studies have investigated these particular issues despite the urgent need for preserving taxa by adequate *ex situ* conservation strategies (Parsons & Hermanutz 2006, Kothera *et al.* 2007, Volis & Blecher 2010, Zhang *et al.* 2010).

Endemic metallophytes are often directly threatened by surface mining activities exploiting economically important metals such as copper, cobalt or zinc (*e.g.,* Bradshaw 2000, Whiting *et al.* 2004, Saad *et al.* 2011). Restoring the habitats after mining exploitation is often not possible and hence the main solution is to conduct *ex situ* conservation and/or to consider plant relocation in protected sites. Nevertheless, the degradation of metal outcrops by mining exploitation may also result the creation of new habitats metalliferous (*e.g.,* Allen & Sheppard 1971, Ginocchio *et al.* 2002, Batty 2005). Such sites can be recolonized by plant species adapted to open habitats and high metal toxicity, and therefore may also serve as substitution sites (*e.g.,* Bradshaw 1983, Ash *et al.* 1994, Bizoux *et al.* 2008, Faucon *et al.* 2009). It might, however, bring formerly spatially isolated congeneric species to co-occur, increasing the risk of competition and hybridization.

In this article we investigated the relationships between two closely related metallophyte species belonging to the Linderniaceae (Rahmanzadeh *et al.* 2005), *Crepidorhopalon perennis* (P.A. Duvigneaud) Eb. Fisch. and *C. tenuis* (S. Moore) Eb. Fisch. (syn.: *Lindernia damblonii* P.A. Duvign.). *Crepidorhopalon perennis* is a narrow endemic pioneer species occurring on copper-rich soils, restricted to only one (formerly two) known site in the vicinity of Lubumbashi, Katanga, Democratic Republic of Congo (Duvigneaud & Denaeyer-De Smet 1963, Brooks & Malaisse 1985). Its restricted distribution, ability to grow on soils with extremely high copper and cobalt concentrations, and capacity to accumulate these metals (Brooks & Malaisse 1985, Reeves & Baker 2000) make *C. perennis* a taxon of high conservation value (Faucon *et al.* 2009, 2010). It is worth emphasizing that this species is critically endangered (Faucon *et al.* 2010) as more than 90 percent of its population has been destroyed, declining from

about 100,000 individuals in 2006 to a few hundred in 2009 (Faucon 2009). Its persistence *in situ* appears to be compromised. Under these critical conditions, particular conservation measures are urgently needed, including the preservation of plants outside its original location. Unfortunately, *ex situ* transplantation attempts in botanical garden (Agronomy Faculty, Lubumbashi University) in 2006–2008 failed because *C. perennis* plants did not survive the drought of the dry season (Kizila 2009). Specific ecological conditions contributing to drought tolerance, such as micro-relief or symbiotic interaction with soil micro organisms may have been lacking. The only sustainable possibility appears to be creating new populations in substitution sites.

In the most suitable substitution sites, *i.e.,* those sites with ecological conditions similar to the species' native site and offering some guarantee for long-term preservation, *C. tenuis* can be found. This species is rare, but has a much broader geographical range, sometimes occurring on non-metalliferous soils (Fischer 1999, Faucon *et al.* 2009). Duvigneaud and Denaeyer-De Smet (1963) suggested that the copper-specialist *C. perennis* might be a derivative of *C. tenuis*. So far, however, information is lacking in terms of their phylogenetic relationships. Given that *Crepidorhopalon tenuis* occurs in the only known *C. perennis* site (Duvigneaud & Denaeyer-De Smet 1963) and that there have been high levels of disturbance because of industrial mining activities for at least more than a century, it is not known if these taxa ever coexisted before mining exploitation. This might have different consequences in terms of long-term preservation of *C. tenuis*. Indeed, no study has investigated yet whether these species occur in sympatry and have overlapping ecological niches (*e.g.,* similar soil requirements and pollinator assemblage), which might lead to inter-specific competition or if they coexist under parapatry, with distinct niches, implying no competition. Moreover, no information regarding their hybridization potential could be found. Before planning any *ex situ* conservation efforts it is essential to investigate how *C. perennis* and *C. tenuis* co-occur. Given the lack of available molecular markers, morphological traits were used for characterizing the two species. In this respect, several questions were addressed, as following: (1) based on the morphometric analysis of vegetative and floral traits can we detect morphologically intermediate hybrid forms between the two species? And, (2) do the ecological niches (based on edaphic conditions and pollinator assemblage) of the two species overlap? Our results will allow us to ascertain whether *C. perennis* may be at hybridization risk and/or in competition with its congener *C. tenuis*.

METHODS

STUDY TAXA—*Crepidorhopalon perennis* (Linderniaceae) is a small, cushion-forming, short-lived perennial herb, producing lilac-blue flowers. This narrow endemic occurs only in one (formerly two) known site (Mine de l'Etoile), where it colonizes disturbed mine deposits rich in copper. *Crepidorhopalon perennis* can flower the whole year, with a flowering peak in March–April. One plant of *C. perennis* produces on average 2227 seeds (ranging from 80 to

4400, $N = 20$) that germinate at the beginning of the rainy season in November. The mean number of seeds per fruit is 30 (ranging from 12 to 61). The mean seed germination rate is 80 percent (M.-P. Faucon, unpubl. data).

Crepidorhopalon tenuis is a small annual herb, with lilac-blue flowers. This species is more common than *C. perennis* as it exists in 24 populations distributed in the Zambezian Regional Center of Endemism (southcentral Africa), of which 18 occur in Katanga (Faucon 2009). It grows on naturally copper-rich soils, but also on substrates (often mine debris) that have been disturbed and reworked by mining activities and on soil contaminated by atmospheric fallout from an ore-smelter foundry (in the Lubumbashi region). *Crepidorhopalon tenuis* has its ecological optimum in pioneer metalliferous habitats, especially on bare or recently disturbed substrates with high Cu contents, where perennial species have not yet established. It has also been occasionally found on non-metalliferous soil, and can therefore be considered as a pseudometallophyte (Fischer 1999, Leteinturier *et al.* 1999, Faucon *et al.* 2009). This species is not threatened and may currently be expanding in the recently created metalliferous habitats as a result of mining activities (Faucon *et al.* 2009). The plants flower from early March to late April, overlapping for 2 mo with *C. perennis.* A single plant can produce on average 1650 seeds (ranging from 40 to 2000) that germinate at the beginning of the rainy season in November. The seeds are dispersed from May to June. In permanently moist habitats, this species can germinate and complete its life cycle throughout the year. The mean number of seeds per fruit is 29 (ranging from 12 to 51). Mean germination rate is 70 percent. Based on flow cytometry, both species have the same ploidy level (Fig. S1). An *in situ* pollination (flower bagging) experiment—almost completely destroyed by mining activities—indicated that both species can produce seeds by selfing (M.-P. Faucon, unpubl. data).

STUDY SITE—The study site was the Mine de l'Etoile (locally known as Kalukuluku), which is situated ~10 km northeast of Lubumbashi (Province of Katanga, Democratic Republic of Congo; 11° 38′ S; 27° 35′ E,. 1277 m asl). This site supports a diverse copper flora (about 150 vascular plant taxa) from Katanga (Duvigneaud & Denaeyer-De Smet 1963, Malaisse & Grégoire 1978). It covers more than 2 km² and offers a broad spectrum of ecological conditions, from recently disturbed mine debris to ancient steppic savanna. Recent revival of mining activity, however, has considerably altered the site and the endemic flora of the site is now endangered. Five sites where *C. tenuis* occurs, located in Katanga province (Dem. Rep. of Congo) were also studied for the assessment of the soil/ecological niche characteristics of this taxon and investigating their potential as substitution sites for *C. perennis.*

MORPHOLOGICAL AND EDAPHIC VARIATION—To examine ecological and morphological variation in *C. perennis* and *C. tenuis,* several traits were measured in the field on fresh plant material for 20 individuals of the population of each species in the one sympatric location at the Mine de l'Etoile. Individuals were randomly sampled in April 2008 along a transect length of 200 m. Identification of *C. perennis* and *C. tenuis* was based on the taxonomic treatment for *Crepidorhopalon* (Fischer 1999). For each individual, 14 morphological characters were measured on three flowers and two leaves (Fig. 1): cauline leaf length (L *l*) and width (L *w*), bract length (B *l*) and width (B *w*), corolla upper lip length (UL *l*) and width (UL *w*), lower lip 1 length (LL1 *l*) and width (LL1 *w*), lower lip 2 length (LL2 *l*) and width (LL2 *w*), lower lip 3 length (LL3 *l*) and width (LL3 *w*), and corolla tube diameter (CT *d*) and length (CT *l*). These variables were used to calculate the following variables expressing plant (leaf, bract and flower) shape: L *l*/L *w*, B *l*/B *w*, UL *l*/UL *w*, LL1 *l*/LL1 *w*, LL2 *l*/LL2 *w*, LL3 *l*/LL3 *w*, and CT *l*/CT *d*. For the 21 variables, mean values per individual were calculated.

To confirm that the observed patterns between species were constant when considering other *C. tenuis* populations, morphological measures (with mean values per individual) were made on three flowers and two cauline leaves from dried herbarium specimens (from BR and BRLU herbaria), for 20 individuals of *C. perennis* from the Mine de l'Etoile and from 40 individuals of *C. tenuis* from 16 metallicolous populations in Katanga. Thirteen variables that could be measured on dry material were obtained:
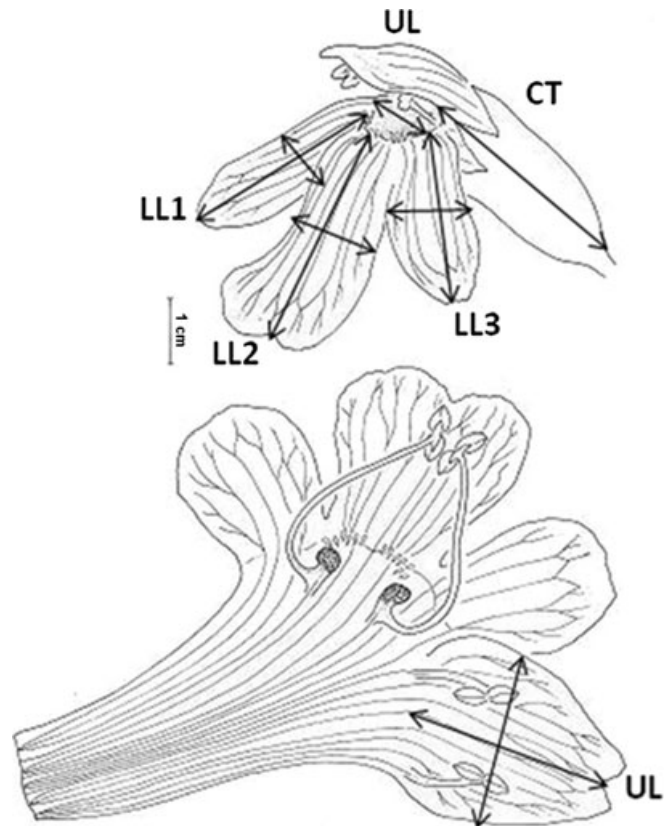

FIGURE 1. Floral characters (maximal lengths and widths) measured in *Crepidorhopalon perennis* and *C. tenuis* (from Fischer 1999). CT: corolla tube; UL: upper lip of corolla; LL: lower lip of corolla; LL1: sub lower lip right; LL2: sub lower lip median; LL3: sub lower lip left.

cauline leaf length (L *l*) and width (L *w*), bract length (B *l*) and width (B *w*), corolla tube length (CT *l*), calyx length (Caly *l*) and width (Caly *w*), capsule length (Caps *l*) and width (Caps *w*), and variables expressing shape (L *l*/L *w*, B *l*/B *w*, Caly *l*/Caly *w*, and Caps *l*/Caps *w*).

To characterize the chemical and physical soil conditions near *C. perennis* and *C. tenuis* plants, 20 soil samples of 100 g each were collected from each site and species from the rhizosphere of each individual (0–10 cm) at Mine de l'Etoile and five other sites where *C. tenuis* occurs. All soil samples were air-dried and sieved using a 2-mm stainless steel sieve. The pH (water) was measured with a glass electrode (VWR symphony SP70C). Organic matter content was measured by loss on ignition at 550°C during 12 h. Mineral elements were extracted with ammonium acetate-EDTA (1M, pH 4.65) for 30 min. (5 g dry soil in 50 ml) (Cottenie *et al.* 1982). The supernatant was then filtered and analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Varian Vista MPX, Mugrave, Australia) for the characterization of the extractable fractions of Ca, Mg, K, P, Cu, Co, Mn, Fe and Zn.

POLLINATOR SAMPLING—To investigate whether *C. perennis* and *C. tenuis* might compete for pollinators, we characterized the pollinator assemblage during peak flowering (April) for both species. Twenty plots of 0.5 × 0.5 m showing 100 percent flowering plant cover were randomly located in the area of the site where both species occurred. Ten plots corresponded to monospecific patches of *C. perennis* and ten plots to *C. tenuis*. The observations were made from 0700 to 1800 h, and were repeated the next day. The observations in monospecific plots of *C. perennis* and *C. tenuis* were made one plot after the other by alternating the species. The order of plot observations was the same for the 2 d. Each day, in each plot, all insects visiting flowers of *C. perennis* and of *C. tenuis* were counted for a period of 20 min. An insect was considered as a visitor when it landed on at least one open flower, for at least 1 s. Captured insects were conserved in 5 percent ethanol and identified subsequently (S. Patiny, University of Liège, Belgium). Most species could not be identified to the species level, but in this case they were well individualized as separate taxa based on their morphology. Mean values of the number of visiting insects were calculated for each plot and each insect taxon as a mean of the observations made for the 2 d.

DATA ANALYSIS—To distinguish between *C. perennis* from *C. tenuis* with respect to leaf and floral morphology, one-way ANOVAs testing for significant differences in morphological traits between species were performed using STATISTICA 8 (Statsoft 2008, Tulsa, OK, U.S.A.). To summarize the patterns of interrelationships of morphological characters, a Principal Component Analysis (PCA) was performed using CANOCO 4.5 (ter Braak & Smilauer 2002). The Bonferroni correction method for multiple comparisons (Rice 1989) was applied when multiple tests were involved.

The potential for hybridization between *C. perennis* and *C. tenuis* was evaluated using the methodology of Parsons and Hermanutz (2006), which is based on an analysis of floral morphological variation. To identify morphologically intermediate individuals, relative distances were computed between all pairs of individuals of *C. perennis* and *C. tenuis* based on ten floral morphological traits (the same floral morphological traits as for ACP). A cluster analysis was performed on the matrix of relative Euclidean distances using the Ward's method (Ward 1963). Differences in floral morphological traits between the two groups identified by the clustering method, corresponding to the two species were tested using the multi-response permutation procedure (MRPP), a non-parametric method (Biodini *et al.* 1988). Cluster analysis and MRPP were performed using PC-ORD v. 4.26 (McCune & Mefford 1999).

The range of edaphic variation of each species was investigated by performing PCA on 11 chemical (pH, organic matter content, Ca, Mg, K, P, Cu, Co, Mn, Fe and Zn) and one physical (% stones) soil properties measured in the vicinity of the 40 *C. perennis* and *C. tenuis* individuals. Because they varied in measurement units and ranges, all morphological and edaphic variables were standardized (ter Braak & Smilauer 2002). All variables were normally distributed.

The differences in number of visited flowers per plot between *C. perennis* and *C. tenuis* were investigated for each insect taxon using non-parametric Kruskal–Wallis tests (data not normally-distributed) with the mean of number of visited flowers per plot as dependent variable and species as grouping variable. Bonferroni corrections for multiple tests were used for assessing significance.

RESULTS

MORPHOLOGICAL VARIATION AND DETECTION OF HYBRIDS—The one-way ANOVAs revealed that the two congeneric species *C. perennis* and *C. tenuis* showed significant differences ($P < 0.0025$ after Bonferroni correction) in morphological traits (Table 1): *C. perennis* had longer but narrower leaves, longer bracts, and longer lower corolla lips 1 and 2 than *C. tenuis*. Axes 1 and 2 of the PCA explained 50 percent of the total variation in the 21 morphological variables (Fig. S2). The variables that best correlated with axis 1 (PC1) were: leaf length: width ratio (L *l*/L *w*; coefficient of correlation $r = -0.67$), length of the three lower lips (LL1 *l*, LL2 *l* and LL3 *l*; $r = -0.89$, -0.78 and -0.86, respectively), bract length ($r = -0.67$), and leaf length ($r = 0.56$). Those that best correlated with axis 2 (PC2) were the width of the three lower lips (LL1 *w*, LL2 *w* and LL3 *w*; $r = -0.87$, -0.84 and -0.87, respectively) and leaf length ($r = 0.46$). *Crepidorhopalon perennis* plants formed a distinct group with negative values on PC1 (Fig. S2), whereas *C. tenuis* plants had positive values on PC1. The PCA showed high intra-specific morphological variation in both species. The range of variation in *C. perennis* was similar to the range observed in *C. tenuis* (Fig. S2). The cluster analysis based on floral traits indicated that the two species were separated into two groups, belonging to two separated clusters (Fig. 2). These groups were significantly different when tested by MRPP (A = 0.207, $P < 0.001$). PCA using 13 morphological variables measured on dried herbarium material of *C. perennis*

TABLE 1. *Morphological characters (mean values, in mm) for* C. perennis *and* C. tenuis *species, and one-way ANOVA tests (F).*

Morphological Characters	C. perennis Mean (SD)	C. tenuis Mean (SD)	$F_{1, 39}$
Leaf length (L l)	11.85 (2.91)	9.28 (2.79)	8.11[*]
Leaf width (L w)	1.75 (0.57)	3.50 (1.41)	26.42[*]
L l/L w	7.10 (1.73)	3.13 (1.96)	46.22[*]
Bract length (B l)	6.53 (1.45)	3.54 (1.45)	42.73[*]
Bract width (B w)	1.03 (0.36)	0.74 (0.38)	6.02 ns
B l/B w	6.74 (1.71)	5.21 (1.98)	6.81 ns
Upper lip length (UL l)	3.29 (0.27)	3.29 (0.31)	1.03 ns
Upper lip width (UL w)	2.86 (0.24)	2.70 (0.25)	4.26 ns
UL l/UL w	1.15 (0.09)	1.22 (0.12)	4.24 ns
Lower lip 1 length (LL1 l)	3.36 (0.33)	3.03 (0.40)	7.92[*]
Lower lip 1 width (LL1 w)	2.18 (0.21)	2.22 (0.26)	0.21 ns
LL1 l/LL1 w	1.54 (0.11)	1.37 (0.13)	19.03[*]
Lower lip 2 length (LL2 l)	3.19 (0.31)	3.03 (0.41)	2.02 ns
Lower lip 2 width (LL2 w)	2.01 (0.19)	2.10 (0.37)	1.07 ns
LL2 l/LL2 w	1.59 (0.12)	1.45 (0.14)	11.11[*]
Lower lip 3 length (LL3 l)	3.21 (0.25)	2.90 (0.33)	11.71[*]
Lower lip 3 width (LL3 w)	2.09 (0.18)	2.11 (0.21)	0.13 ns
LL3 l/LL3 w	1.54 (0.09)	1.38 (0.14)	17.40[*]
Corolla tube diameter (CT d)	2.59 (0.24)	2.60 (0.22)	0.01 ns
Corolla tube length (CT l)	5.24 (0.21)	5.16 (0.35)	0.80 ns
CT l/CT d	2.03 (0.16)	1.99 (0.18)	0.60 ns

[*]$P < 0.0025$ after Bonferroni correction, ns = not significant.

from the Mine de l'Etoile and of *C. tenuis* from 16 metallicolous populations showed the same pattern of species clustering into two distinct groups (Fig. S3).

ECOLOGICAL NICHE VARIATION (SOIL AND POLLINATOR ASSEMBLAGE)—Axes 1 and 2 of the PCA on 11 chemical (pH, organic matter content, Ca, Mg, K, P, Cu, Co, Mn, Fe and Zn) and one physical (percentage of stones) soil properties explained 48 percent of the total variation (Fig. S4). The variables that best correlated with axis 1 (PC1) were Co, Cu, Mn, pH and P (positive correlation, $r \geq 0.70$), and with axis 2 (PC2) were K, Ca, Mg and organic matter (positive correlation, $r \geq 0.65$). The individuals of *C. perennis* showed higher scores on PC1 (*i.e.*, higher Cu and Co contents) than *C. tenuis*. There was niche overlap (about 80%) between the two species with respect to soil chemical conditions. *Crepidorhopalon perennis* showed an ecological range as broad as *C. tenuis* (Fig. S4).

Ten different insect species were observed visiting *C. perennis* and *C. tenuis*, which belong to eight taxonomic families and three orders (Table 2). The most heavily represented order and family was Hymenoptera: Sphecidae with three species (Table 2). *Ammophila* appeared to be the most abundant genus observed on *C. perennis* and *C. tenuis* flowers, with an average range of 0.12 to 0.71 visits per plot per 20 min. Three other species of Hymenoptera were frequent (up to 8 visits per plot per 20 min): *Amegilla sp.* (Apidae), *Bembix sp.* (Crabronidae) and *Lasioglossum sp.* (Halictidae). *Crepidorhopalon perennis* and *C. tenuis* were visited by the ten same species. No significant difference in the number of visits by insects was found between the two species (Kruskal–Wallis test: $H < 1$, $P > 0.05$).

DISCUSSION

CREPIDORHOPALON PERENNIS AND C. TENUIS, TWO DISTINCT MORPHOLOGICAL ENTITIES—The traits used in the literature to distinguish *C. perennis* and *C. tenuis* are (1) the contrasting life cycle (perennial vs. annual, respectively) and; (2) the number of basal leaves, which is higher in *C. perennis* (Fischer 1999). Although a close morphological resemblance was emphasized by several studies (Duvigneaud & Denaeyer-De Smet 1963, Brooks & Malaisse 1985), none were based on quantitative trait measurements. Our results show, for the first time, that although the two species have very similar appearance, they can be easily defined based on a combination of morphological traits. Significant morphological differences exist between the two species, especially in leaf length and width, in bract length and in the length of the lower corolla
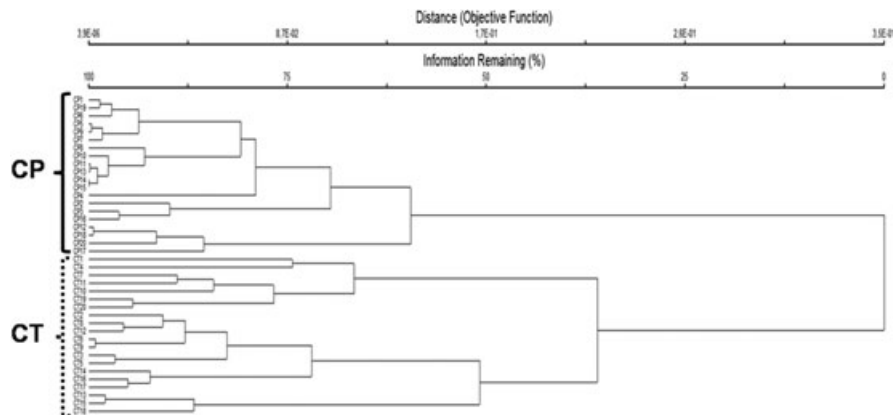

FIGURE 2. Ward's dendrogram of the cluster analysis on floral characters for 20 individuals of *Crepidorhopalon perennis* (CP) and of *C. tenuis* (CT).

TABLE 2. *Number (N) of visits per 20 min (mean value with SD in the brackets) for each insect taxon in 10 plots of* C. perennis *and* C. tenuis. *Kruskal–Wallis tests were not significant (P > 0.05).*

Order	Family	Taxon	N visits (SD) C. perennis	N visits (SD) C. tenuis
Hymenoptera	Sphecidae	*Ammophila species 1*	0.71 (1.23)	0.41 (0.85)
Hymenoptera	Sphecidae	*Ammophila species 2*	0.12 (0.46)	0.25 (0.55)
Hymenoptera	Formicidae	*species 3*	0.05 (0.21)	0.12 (0.86)
Hymenoptera	Apidae	*Amegilla sp.*	0.55 (1.33)	0.27 (0.61)
Hymenoptera	Crabonidae	*Bembix sp.*	0.42 (1.82)	0.23 (0.55)
Hymenoptera	Sphecidae	*Podalonia sheffieldi*	0.05 (0.23)	0.04 (0.23)
Hymenoptera	Halictidae	*Lasioglossum sp.*	0.35 (1.37)	0.21 (0.73)
Hymenoptera	Crabonidae	*Tachysphex sp.*	0.05 (0.23)	0.05 (0.32)
Lepidoptera	Nymphalidae	*Acraea pseudolycia*	0.05 (0.25)	0.05 (0.29)
Diptera	Syrphidae	*species 4*	0.05 (0.21)	0.45 (1.64)

lips. Some of these may have an adaptive significance: for instance, leaves are much wider and therefore their area is about three times higher for *C. tenuis* (mean value = 3.2 cm²) than for *C. perennis* (mean value = 1.9 cm²). This difference might be explained by their annual or perennial life history traits and their respective growth season. Indeed, because of their short life cycle, higher leaf area can be expected in annual plant species compared with perennial congeners (Garnier 1992). The narrower leaves of *C. perennis* may also represent an adaptation to drought (Turner 1986), as *C. perennis* grows during both wet and dry seasons, whereas *C. tenuis* plant growth takes place mainly during the wet season. Besides, *C. perennis* has a longer flowering period than *C. tenuis*, and may therefore be visited by other pollinator guilds. Difference of pollinator guilds might have contributed to fix the differences in the corolla shape and size (Fenster *et al.* 2004).

COHABITATION OR HYBRIDIZATION?—The two species appear to grow in sympatry rather than in parapatry: there is a niche overlap between the two species, and individuals of both populations may be spatially intermixed. *Crepidorhopalon perennis*, however, occurs mostly in the most contaminated and alkaline soils (higher concentrations of Co, Cu and pH) and *C. tenuis* in more fertile soils (higher contents of Ca, K, Mg and organic matter), suggesting that there might be an ecological segregation at a very fine scale. Moreover, from our study it appears that they share the same pollinator assemblage, suggesting that pollen transfer might occur between the two species. This could potentially result in pollen waste and clogging by heterospecific pollen for the rarer species (Kwak *et al.* 1998, Wilcock & Neiland 2002). These findings, combined with a two-month overlapping flowering period, might favor interspecific crossings and hybrid appearance. Yet no evidence of hybridization was found between *C. perennis* and *C. tenuis*, as no morphologically interme-

diate individuals could be identified, even under conditions suited for hybridization. Therefore, our failure to detect hybrids suggests that the two species might not be inter-fertile, resulting from prezygotic reproductive isolating mechanisms (Levin 1971, Pascarella 2007), perhaps through pollen-stigma incompatibility. Differences in floral features, such as corolla shape or size, may differentially attract pollinators and also contribute to reproductive isolation (*e.g.,* Gardner & Macnair 1999, Rajakaruna 2004). Despite the longer flower lips found for *C. perennis*, however, no difference in pollinator assemblage was observed between the two species.

Postzygotic mechanisms, such as hybrid seed abortion, seedling inviability and adult sterility, may also lead to reproductive isolation (Ramsey *et al.* 2003, Taylor *et al.* 2009). Hybrid plant fitness may be reduced in either parental habitat compared with the two species (Van Rossum *et al.* 1996, Rieseberg & Carney 1998, Arnold *et al.* 2008). The drought of the dry season (May–October) in Katanga might represent a strong selection pressure against hybrids between *C. perennis* and *C. tenuis*, as one of the parental species (*C. tenuis*) mainly grows during the wet season and has wider leaves, less adapted to drought. Hybrids, however, might not always be morphologically intermediate (Abbott & Low 1996, Rieseberg & Carney 1998). They might also be diluted in the many descendants of *C. perennis* that may be produced outside the flowering of *C. tenuis* (May–February). Our results certainly deserve further investigation for confirmation, *e.g.,* by crossing experiments between the two species, by testing within- and between-species pollen-stigma compatibility and using molecular markers for hybrid detection.

IMPLICATIONS FOR CONSERVATION MANAGEMENT—The endemic *C. perennis* is critically endangered because the area occupied by its population has been considerably reduced by intensive mining. From 100,000 individuals recorded in 2006 only a few hundred still exist nowadays (Faucon 2009). This is a critical situation that requires urgent conservation measures and may justify population translocation (IUCN World Conservation Union 2002, Guerrant *et al.* 2004, Maunder *et al.* 2004b). The *ex situ* conservation efforts consisting in keeping a living collection of plants in the botanical garden at Lubumbashi University failed, likely because of the difficulty for recreating suitable ecological conditions for the taxon, *e.g.,* micro-relief and top soil conditions (Kizila 2009). The introduction of a population to a substitution site seems the only solution for the conservation of this taxon. Such management of a threatened species requires precise species delineation, including studies of its biology and restoration potential. Our data indicate that *C. perennis* conservation program could be carried out without necessarily taking the presence of *C. tenuis* into account. The lack of hybridization with its congener, *C. tenuis,* allows the introduction of *C. perennis* to ecologically suitable sites, where *C. tenuis* often occurs. Our soil data (Fig. S4) suggest that Niamumenda and Kalabi populations might be good candidates. Whether the sharing of pollinators contributes to gene exchange between the two species or result in competition for pollinators, however, certainly requires further investigation. This is essential for properly

assessing the minimal population sizes needed for establishment. The apparently wide ecological amplitude of *C. perennis*, and its ability to colonize anthropogenic copper-rich habitats, indicates that *C. perennis* could succeed in other metalliferous habitats outside its native site.

PERSPECTIVES—To propose an adequate *ex situ* conservation program further studies may be pursued. Firstly, it is necessary to confirm the absence of a hybridization risk between the two species and to know more about their reproductive systems. Crossing experiments and the study of the genetic diversity and structure using molecular markers may allow the estimation of the selfing and outcrossing rates, assessing pollen compatibility between species and detecting if gene exchange exists between species by identifying hybrids. Secondly, before starting any large-scale introduction of plants in substitution sites, it is essential to confirm that this species is able to grow outside its native site, by preliminary transplantation experiments, using seeds and/or plants. Finally, it is necessary to identify the key factors for the long-term persistence of the newly created populations, *e.g.,* by long-term studying the dynamics of the population demographic, and by investigating seed dispersal ability of species and pollination processes.

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SUPPORTING INFORMATION

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

FIGURE S1. Flow cytometry data of individuals of *Crepidorhopalon perennis* and *C. tenuis* from Mine de l'Etoile.

FIGURE S2. Principal components analysis biplot of 21 morphological variables for 20 individuals of *Crepidorhopalon perennis* and of *C. tenuis*.

FIGURE S3. Principal components analysis biplot of 15 morphological variables for 20 individuals of *Crepidorhopalon perennis* and for 40 individuals of *C. tenuis* from 16 metalliferous sites in Katanga.

FIGURE S4. Principal components analysis biplot of 11 edaphic variables measured for *Crepidorhopalon perennis* population and for six *C. tenuis* populations.

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