



2 **A preliminary assessment of the genetic structure of the invasive plant**
3 ***Cortaderia selloana* (Poaceae) in the Iberian Peninsula**

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7 **Abstract**

8 The proliferation of invasive plants in ecosystems is one of the main causes of biodiversity loss. These invasive plants not
9 only negatively affect native species but also influence the environment, causing economic and public health damage to
10 humans. The control and eradication of these plants is an essential strategy for the conservation of native species. In the
11 present study, we report the first molecular-level data for the invasive plant *Cortaderia selloana* (pampas grass) in the Iberian
12 Peninsula. Our results indicate that the use of microsatellites is a highly recommended tool to establish patterns of genetic
13 variation in this invasive species. The techniques used (chloroplast DNA sequencing and microsatellites) can be the starting
14 point for analyzing the global diversity of the species and is the prevalence and extent of local adaptation among invasive
15 plant species in comparison to native plant species in the south of Europe.

16 **Keywords** Biodiversity · Conservation · *Cortaderia selloana* · Invasive plant · Molecular study · Prevention

17 **Introduction**

18 At present, endemic species are increasingly threatened due
19 to changes in land use, along with the introduction of exotic
20 species with invasive potential (Domènech 2005; Domènech
21 et al. 2005; Flory and Lockwood 2020; Pyšek et al. 2020;
22 Borden and Flory 2021). In our highly developed society, the
23 human being has become the most efficient seed disperser,
24 and consequently, exotic plant introduction strongly relies
25 on anthropogenic causes. According to Mack and Lonsdale
26 (2001), there are mainly three seed movements: i) accidental
27 movement, when the introduction is not intended; ii)
28 utilitarian movement, when a species is introduced in order
29 to confer a benefit for the population; and iii) ornamental
30 movement, when a species is used as an ornamental plant,

producing great risks of biological invasions. Some of the
introduced plants can quickly adapt to their new environ-
ment, displacing native species (Pyšek et al. 2012).

A good example of biological invasion is *C. selloana*
(Schult. & Schult.f.) Asch. & Graebn, a perennial alien
grass that has become one of the most aggressive invasive
species in southern European areas (Harradine 1991; Lam-
brinos 2001; Domènech and Vilà 2007, 2008; Aguiar and
Ferreira 2013; Vourlitis and Kroon 2013), particularly in
northwest Spain (Domènech et al. 2005; Fagúndez and Bar-
rada 2007; Campos and Herrera 2009; Fernández Rojo et al.
2015; Cires and Fernández Prieto 2017; Ministerio para la
Transición Ecológica 2018; Pardo-Primoy and Fagúndez
2019). *C. selloana*, referred to by the common name pam-
pas grass, occurs mainly in altered habitats associated to
human perturbations (Domènech et al. 2005), with natural-
ized populations steadily spreading from the 1980s through-
out the Cantabrian range (i.e. Galicia, Asturias, Cantabria
and Basque Country; see Sanz-Elorza et al. 2004; Ministerio
para la Transición Ecológica 2018). The main issues con-
cerning *C. selloana* can be summarized as follows: i) once
the plant population becomes established, the habitats are
drastically transformed (e.g. dominant species, pyrophytic
plant, etc.) especially when located in river areas, banks,
dunes and estuary tails (Sanz-Elorza et al. 2004), and ii)

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56 the pollen produced by *C. selloana* is allergenic (Falagi-
57 ani 1989), which might cause a serious problem for public
58 health.

59 From a reproductive point of view, *C. selloana* repro-
60 duces sexually releasing large amounts of very light seeds
61 (over 800,000 according to Saura-Mas and Lloret 2005),
62 well-adapted to long distance by wind-dispersion (anemo-
63 chory), which makes its dissemination practically uncontrol-
64 lable. It should be noted that *C. selloana* is gynodioecious,
65 meaning that some individuals have female flowers, while
66 others have hermaphrodite ones (Herrera and Campos 2006).

67 Taxonomy of the genus *Cortaderia* has been recently
68 revised (Testoni and Linder 2017), including *C. selloana*
69 among other species (Acevedo de Vargas 1959; Testoni and
70 Villamil 2014). From a molecular point of view, Barker et al.
71 (2003, 2007) suggested that *Cortaderia* is a paraphyletic
72 genus. The phylogenetic relationships inferred from DNA
73 sequence variation in nuclear internal transcribed spacer
74 (ITS) region and the chloroplast *rpoC2* gene, showed that
75 *C. selloana*, *C. jubata* and *C. rudiusscula* belonged to one
76 clade (*Cortaderia* from South America), while *C. fulvida*, *C.*
77 *richardii* and *C. toetoe* were part of the Australasian clade. In
78 addition, to characterize pampas grass cultivars, microsate-
79 lite primers were developed and successfully applied to dis-
80 tinguish different taxa (Ahmad et al. 2006). Unfortunately,
81 little is known about the genetic variability and history of
82 *C. selloana* in the Iberian Peninsula. The main goals of this
83 work are i) to validate the use of nuclear microsatellites in
84 Iberian samples to identify the native geographical origins
85 of invasive populations, and ii) to examine intraspecific pat-
86 terns of chloroplast DNA (cpDNA) of hermaphrodites indi-
87 viduals compared to unisexual (female flowers) individuals.

Material and methods

Plant material and DNA extraction

90 Fresh leaf tissue was collected from 15 plants from four
91 sites located in the regions of Asturias and Galicia (north-
92 west of Spain) (Fig. 1; Table S1). Morphological differences
93 based on reproductive structures (Connor 1973; Herrera and
94 Campos 2006) were used to characterize hermaphrodite and
95 female flowers (Fig. 2). Voucher specimens were collected,
96 dried by pressing in absorbent paper, stored at room tem-
97 perature, and kept in the Herbarium of the University of
98 Oviedo (FCO). Samples for molecular analyses were dried
99 in silica gel and stored before DNA isolation. DNA was
100 extracted using the DNeasy Plant Mini Kit system (Qiagen
101 Inc., Valencia, CA, USA), according to the protocol rec-
102 ommended by the manufacturer. DNA concentration was
103 measured by a Beckman-Coulter DU800® spectrophotom-
104 eter (Fullerton, CA, USA).

Microsatellite markers

106 Three *C. selloana* specific primer pairs were used to geno-
107 type individuals: Cor-7, Cor-11 and Cor-36, as described
108 by Ahmad et al. (2006). Separation of PCR products were
109 performed on an agarose gel (5%) after digestion with *TruI*
110 enzyme (Thermo Scientific) following manufacturer's
111 instructions.

Plastid sequencing and phylogenetic analyses

113 PCR reactions were performed following Cires et al. (2018).
114 Standard primers were used for amplification and sequencing

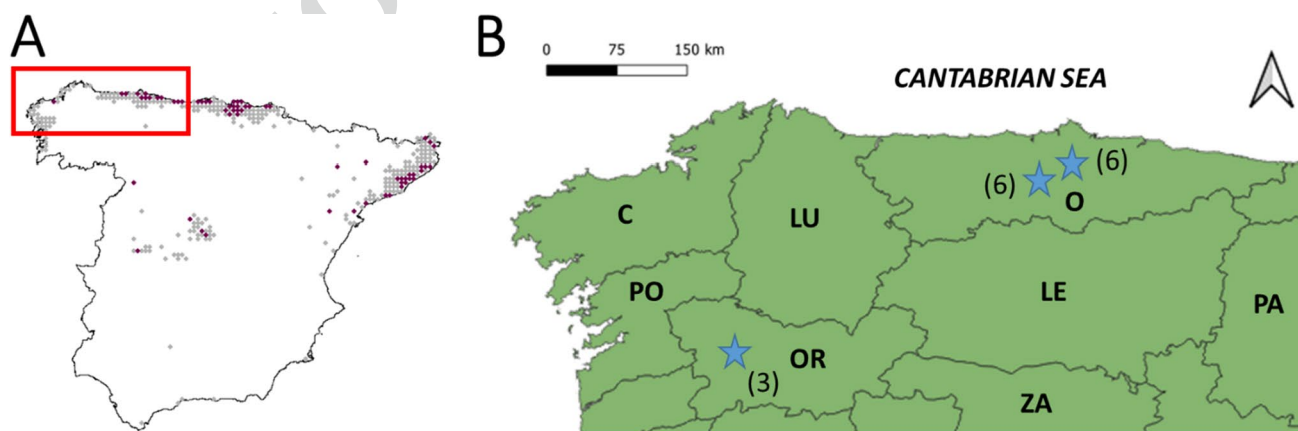
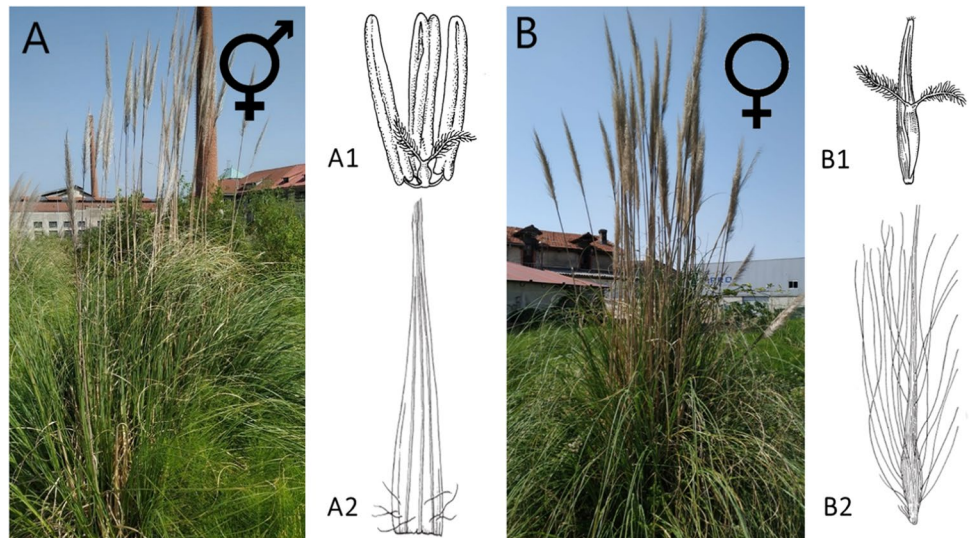


Fig. 1 Distribution area of *Cortaderia selloana* in the Iberian Peninsula (A) and geographical location of the populations analyzed (B). Map data from Spain are based on the actual distribution range (purple) and the potential distribution range (grey) of Gassó et al.

(2012). Blue stars correspond to sampling points. Software: QGIS 3.8 Zanzibar. Abbreviation of the Spanish provinces: C (A Coruña), LE (León), LU (Lugo), Asturias (O), OR (Ourense), PA (Palencia), PO (Pontevedra), ZA (Zamora)

Fig. 2 Individuals of *Cortaderia selloana*. **A)** size of the plant with hermaphrodite flower, A1) gynoecium and androecium of the hermaphrodite flower, A2) hermaphrodite flower lemma; **B)** size of the plant with female flower, B1) female flower, B2) female flower lemma



115 of plastid sequences (*trnL-F*, Taberlet et al. 1991; *psbA-*
 116 *trnH*, Shaw et al. 2005). PCR products were sequenced at the
 117 DNA Synthesis and Sequencing Facility Macrogen (Madrid,
 118 Spain). Sequence data were assembled using ClustalW and
 119 edited with Geneious 7 (Kearse et al. 2012). The obtained
 120 sequences are deposited in GenBank (see Table S1 for acces-
 121 sion numbers). The sequence of *Trisetum bifidum* was used
 122 as outgroup. Phylogenetic analyses were performed using
 123 Maximum Parsimony (MP) and Maximum Likelihood (ML)
 124 methods and conducted by a heuristic search using MEGA
 125 X (Kumar et al. 2018). The MP consensus tree was obtained
 126 using the Tree-Bisection-Regrafting (TBR) algorithm with
 127 search level 5, in which the initial trees were obtained by
 128 the random addition of sequences (1000 replicates). On the
 129 other hand, ML consensus tree was inferred using Kimura
 130 2-parameter model (Kimura 1980). In both phylogenetic
 131 approaches, bootstrap consensus tree was inferred from 1000
 132 replicates and branches corresponding to partitions repro-
 133 duced in less than 50% bootstrap replicates were collapsed.

134 **Results and discussion**

135 The invasion of *C. selloana* causes displacement in native
 136 flora, ecosystem alterations, degradation of the landscape
 137 and loss of biodiversity (Ministerio para la Transición
 138 Ecológica 2018). Despite being one of the main invasive
 139 plant species in the Iberian Peninsula (Gassó et al. 2012;
 140 Sanz-Elorza et al. 2004; Tarabon et al. 2018), there are still
 141 few molecular studies to date (see Barker et al. 2003, 2007;
 142 Ahmad et al. 2006; Okada et al. 2007; Pirie et al. 2008).

143 In the present work, two molecular methods have been
 144 used for a preliminary evaluation of the diversity of *C.*
 145 *selloana*. Information on genetic diversity patterns can
 146 contribute to reduce the number of populations that need

to be controlled to impede the spread of the species, and 147
 also provides an evolutionary and demographic view of the 148
 taxon (Milligan et al. 1994). Our results show the suitability 149
 of using those microsatellites developed by Ahmad et al. 150
 (2006) for different samples of *C. selloana* from the Iberian 151
 Peninsula. The three selected microsatellites (Cor-7, Cor- 152
 11 and Cor-36) showed molecular diversity of the isolated 153
 populations (Galicia) versus the core of the populations ana- 154
 lysed (Asturias). It is noteworthy that, when different types 155
 of individuals (female and hermaphrodites) were analyzed, 156
 no differences were found (data not shown). We are aware 157
 of the limitations of using microsatellites and their visuali- 158
 zation in agarose gels, but the objective of this preliminary 159
 pilot study was simply to verify that the use of these mark- 160
 ers is effective in samples of European pampas grass. On 161
 the other hand, two plastid DNA barcode markers have also 162
 been used (Fig. 3). Despite the reduced sample size in this 163
 study, our results indicate that invasive *C. selloana* contains 164
 very low haplotype differentiation: only the absence of an 165
 adenine (poly-A region) in 6 samples was found for the *trnL-*
F intergenic spacer. In addition to this, no differences were 166
 found within the 577 bp of *psbA-trnH*. 167
 168

Previous studies (e.g. Okada et al. 2007; Houliston 169
 and Goeke 2017) have shown that certain clones of *C.* 170
selloana have a greater invasive capacity. For example, in 171
 New Zealand there are two differentiated genetic groups 172
 with a variable degree of invasion, although how these two 173
 groups ecologically vary is unclear, and requires further 174
 studies (Houliston and Goeke 2017). Given the negative 175
 influences on human health, such as allergy phenomena 176
 (e.g. Fernández Rojo et al. 2015; Fernández Prieto and 177
 Valderrábano Luque 2017), it will be important to carry 178
 out a larger study at national level to know the degree 179
 of invasiveness of the different identified populations 180
 throughout the Iberian Peninsula. Also, future studies will 181

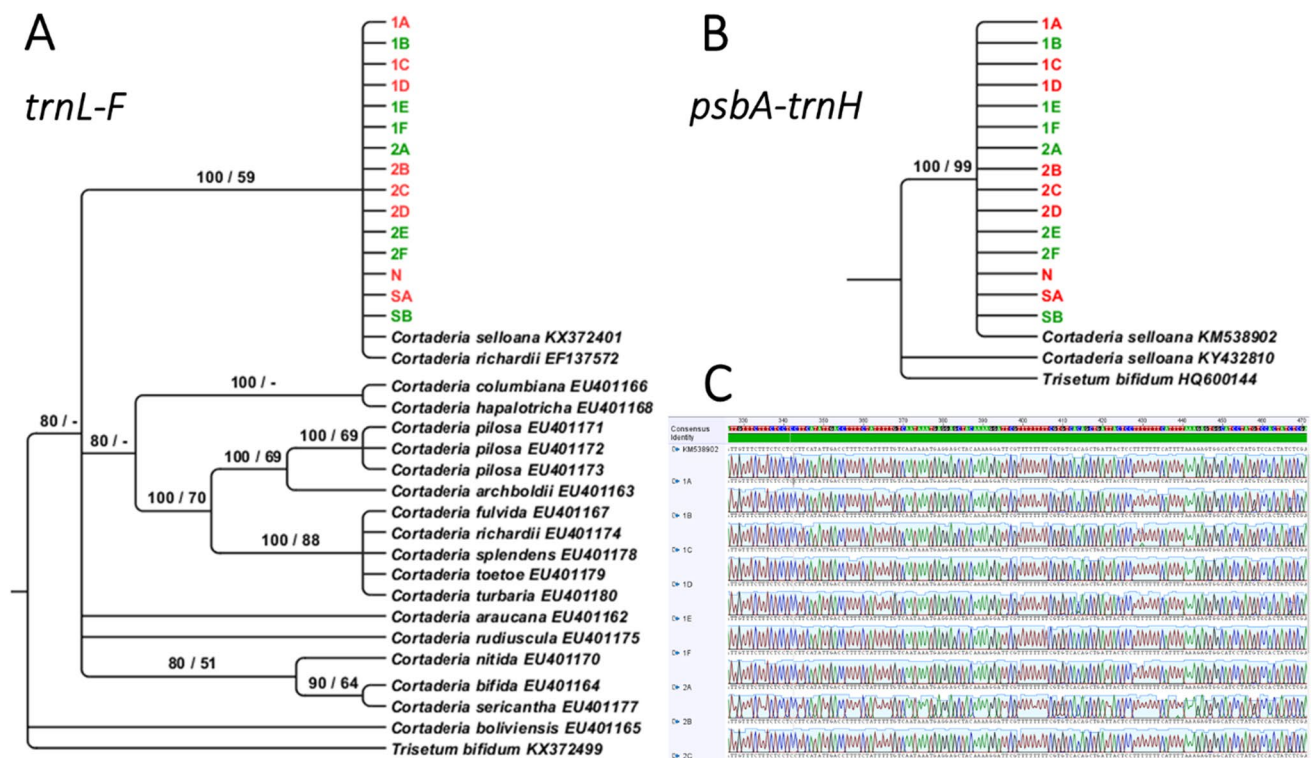


Fig. 3 Consensus phylogenetic tree retrieved from MP and ML analysis based on *trnL-F* (A) and *psbA-trnH* (B) of *Cortaderia selloana* sequences. The numbers over the branches correspond to MP / ML bootstrap values. Female plant (in red) and hermaphrodite (in green)

clades are represented. A dash means that the node is not present in the corresponding tree. Number of accession retrieved from GenBank are indicated in italics. C) Example of the quality and similarity of the chromatograms analyzed for different samples of *C. selloana*

182 be necessary to elucidate the relative importance of a field
 183 environment versus a controlled environment to influence
 184 the detection of local adaptation (or lack thereof) in inva-
 185 sive plants versus native plants.

186 Information on the diversity and spatial distribution of
 187 clones of an invasive plant is crucial for understanding its
 188 clonal structure and invasive history (e.g. Ren and Zhang
 189 2007; Wrzesień and Denisow 2017; Byun et al. 2018).
 190 Unfortunately, for many invasive plants, these data are still
 191 unknown, as it is the case of *C. selloana*, which poses
 192 considerable threats to the Iberian Peninsula ecosystem.
 193 Therefore, it would seem wise to develop and implement
 194 control programs to investigate the presence of different
 195 clones, connected to their invasive potential, assisting then
 196 on reducing the severity of these threats. Based on our
 197 results, we recommend the use of nuclear microsatellites
 198 to characterize putative clones within and among popu-
 199 lations of pampas grass, in order to identify those culti-
 200 vars with the greatest invasive capacity and understand
 201 their genetic diversity. This fact should allow, since the
 202 resources of the administrations (Spanish local govern-
 203 ment) are limited, to act and eradicate those nuclei of *C.*
 204 *selloana* that pose a greater environmental threat due to
 205 their high degree of invasive capacity.

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Declarations 215

Conflict of interest On behalf of all authors, the corresponding author 216
 217 states that there is no conflict of interest.

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