

Short Communication

Flow cytometry reveals that the rust fungus, *Uromyces bidentis* (Pucciniales), possesses the largest fungal genome reported—2489 Mbp

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SUMMARY

Among the Eukaryotes, Fungi have relatively small genomes (average of 44.2 Mbp across 1850 species). The order Pucciniales (Basidiomycota) has the largest average genome size among fungi (305 Mbp), and includes the two largest fungal genomes reported so far (*Puccinia chrysanthemi* and *Gymnosporangium confusum*, with 806.5 and 893.2 Mbp, respectively). In this work, flow cytometry was employed to determine the genome size of the *Bidens pilosa* rust pathogen, *Uromyces bidentis*. The results obtained revealed that *U. bidentis* presents a surprisingly large haploid genome size of 2489 Mbp. This value is almost three times larger than the previous largest fungal genome reported and over 50 times larger than the average fungal genome size. Microscopic examination of *U. bidentis* nuclei also showed that they are not as different in size from the *B. pilosa* nuclei when compared with the differences between other rusts and their host plants. This result further reinforces the position of the Pucciniales as the fungal group with the largest genomes, prompting studies addressing the role of repetitive elements and polyploidy in the evolution, pathological specialization and diversity of fungal species.

Keywords: *Bidens pilosa*, flow cytometry, genome size, *Uromyces bidentis*.

Genome size in Eukaryotes varies greatly, as a result of polyploidization and/or structural chromosomal rearrangements. Genome size and its dynamics have direct implications on the evolutionary fitness, reproduction and non-sexual mechanisms for diversity creation (D'Hondt *et al.*, 2011). Among Fungi, previous studies have revealed that genomes vary from <1 to 893 Mbp (Kullman *et al.*, 2005; Tavares *et al.*, 2014). Recently, rust fungi

(Basidiomycota, Pucciniales) have been recognized as the order with the largest average genome size (305 Mbp) (Tavares *et al.*, 2014), with species with genomes as large as 806.5 and 893.2 Mbp (*Puccinia chrysanthemi* Roze and *Gymnosporangium confusum* Dietel, respectively; Tavares *et al.*, 2014). Genome sequences available for a few rust fungi have shown that these genomes are populated with transposable elements and other non-coding repetitive sequences (Cantu *et al.*, 2011; Duplessis *et al.*, 2011; Nemri *et al.*, 2014; Tan *et al.*, 2014). As most of the genome sequences available for rusts represent relatively small genomes, it is expected that larger rust genomes could comprise even higher proportions of such repetitive elements, which is corroborated by preliminary data obtained from *Hemileia vastatrix* Berk. & Broome (Cristancho *et al.*, 2014) and *Uromyces fabae* de Bary ex Cooke (Link *et al.*, 2014). Polyploidy could also account for these large genomes, but no evidence supporting its occurrence has been gathered.

The objective of this work was to employ flow cytometry to determine the genome size of the *Bidens pilosa* L. rust fungus *Uromyces bidentis* Lagerheim, showing that this organism has the largest fungal genome ever reported.

Bidens pilosa L. (black-jack, or Spanish needle, among other common names) is an Asteraceae species originating from the Americas, being widely disseminated in tropical and temperate regions of the world. The genome size of this species has been estimated as $2C = 3325$ Mbp (Bennett and Leitch, 1997). Plants exhibiting rust symptoms were identified and collected at Tapada da Ajuda, Lisbon, Portugal. Cross-sections of infected leaves (20–25 μm) were stained with cotton blue for the observation of fungal structures, or with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) and diethanol (Uvitex 2B, Advanced Technology & Industrial Co., Hong Kong, China) for the visualization of nuclei and fungal cell walls (respectively), as described previously (Tavares *et al.*, 2014). Observations were made with a Leica DM-2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany), under bright field or ultraviolet (UV)

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illumination (excitation filter BP 340–380; barrier filter LP 430). For comparison purposes, nuclei from *Coffea arabica* L. leaves infected with *H. vastatrix* and of *Avena sterilis* L. infected with *Puccinia coronata* Corda were also analysed using the same procedure.

The genome size of the rust and host species was estimated by flow cytometry using a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany). For this, nuclei from rust and host species, together with those of *Solanum lycopersicum* L. 'Stupické' (internal reference standard; $2C = 1.96$ pg or 1917 Mbp; Doležel *et al.*, 1992), were isolated by simultaneously chopping infected leaves of *B. pilosa* with leaves of the reference

standard, as described previously (Loureiro *et al.*, 2007; Tavares *et al.*, 2014). The nuclear suspension was then filtered through a 30- μ m nylon filter to remove plant and fungal debris, and 50 μ g/mL of propidium iodide (Fluka, Buchs, Switzerland) and 50 μ g/mL of RNase (Fluka) were added to stain DNA only. Data were acquired using Partec FloMax software v2.4d (Partec GmbH), as described previously (Tavares *et al.*, 2014). Fluorescence peaks of fungal nuclei were identified by comparing fluorescence histograms of rust-infected leaves and of healthy *B. pilosa* leaves.

Rust symptoms were observed on leaves of flowering *B. pilosa* plants between May 2014 and January 2015 (Fig. 1a,b). The symp-

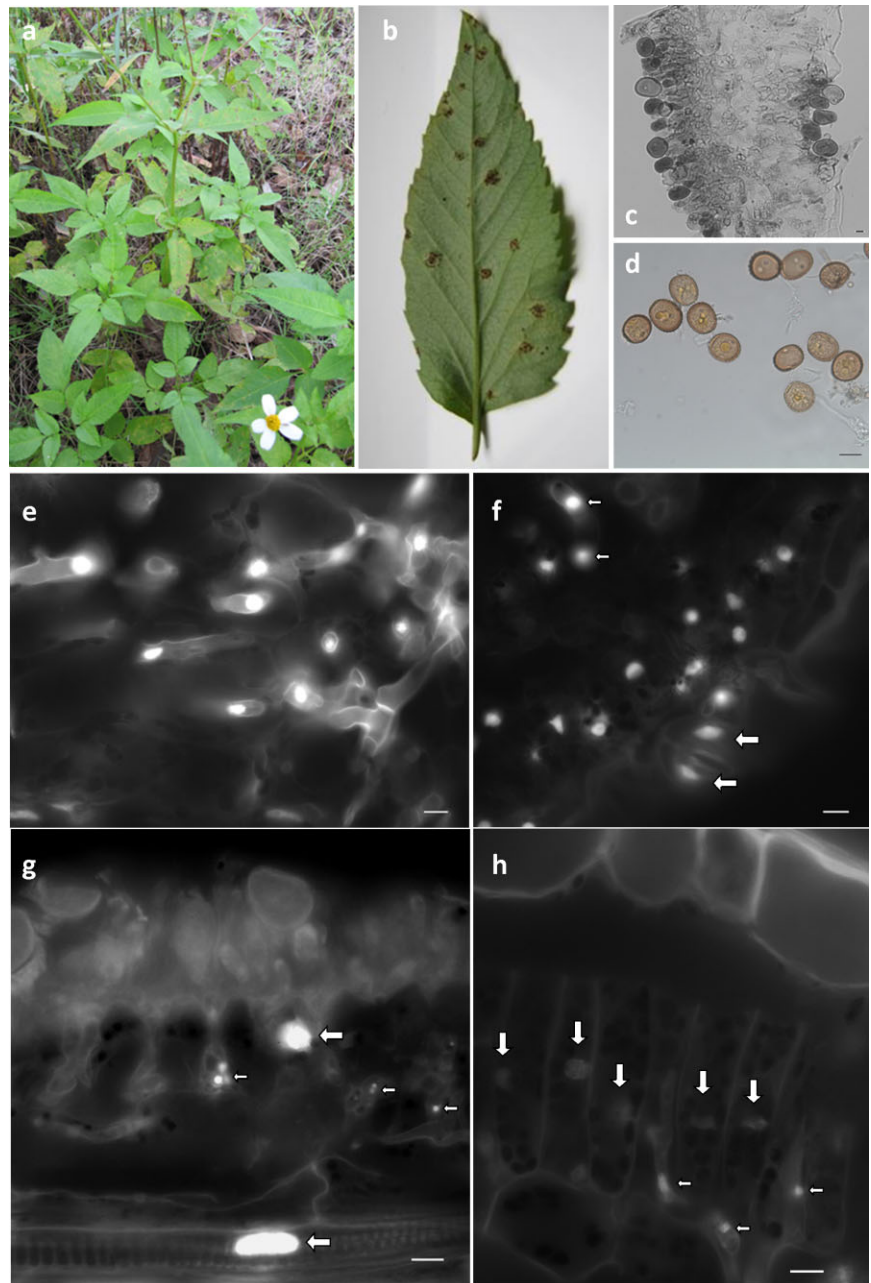


Fig. 1 *Uromyces bidentis* symptoms and structures, and nuclei by comparison with nuclei of other organisms. (a) Symptoms in *Bidens pilosa* plants in the field (Tapada da Ajuda, Lisbon, Portugal; November 2014). (b) Uredinia on the abaxial surface of *B. pilosa* leaflet. (c) Cross-section of an infected leaf showing uredinia. (d) Urediniospores. (e–h) 4',6-Diamidino-2-phenylindole (DAPI)-stained nuclei (bar, 10 μ m; small arrow, fungal nuclei; large arrow, plant nuclei), visualized under fluorescence, of *U. bidentis* (e), of *U. bidentis* and *Bidens pilosa* including stoma (f), of *Puccinia coronata* and *Avena sterilis* (g) and of *Hemileia vastatrix* and *Coffea arabica* (h).

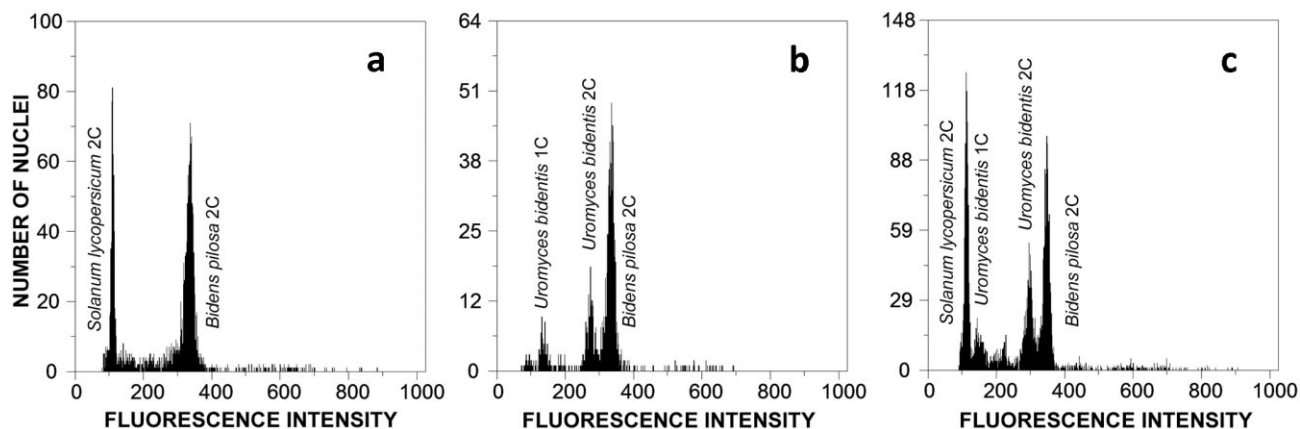


Fig. 2 Flow cytometric histograms of relative fluorescence intensities of propidium iodide-stained nuclei isolated from healthy *Bidens pilosa* leaves and the plant DNA reference standard *Solanum lycopersicum* (a), *Uromyces bidentis*-infected *B. pilosa* leaves (b), and *U. bidentis*-infected *B. pilosa* leaves and the plant DNA reference standard *S. lycopersicum* (c).

toms began with the appearance of brownish spots on both sides of *B. pilosa* leaves. On the abaxial surface of the leaves, the spots gradually became raised, and distinct circular light-brown uredinia, first subepidermal then erumpent, appeared on the spots. Premature leaf chlorosis, senescence and defoliation were common on leaves where sori were abundant. Microscopic examination of urediniospores and sori (Fig. 1c,d) indicated that the pathogen is *Uromyces bidentis* Lagerheim, *Bull. Soc. Myc. Fr.* **11**, 213, 1895 (= *U. bidenticola* Arth., *Mycologia*, **9**, 71, 1917) (Baker, 1955; Sydow, 1910). A herbarium specimen was deposited in the 'João de Carvalho e Vasconcellos' herbarium (LISI), under accession LISI-FUNGI-00026.

At the nuclear level, it has already been shown that genome size is correlated with both nuclear (Baetcke *et al.*, 1967) and chromosome (Bennett *et al.*, 1983) volume. Therefore, the larger the genome size, the greater the minimum nuclear volume. Considering that the microscopic examination of infected leaf sections revealed that the fungal nuclei were not very different in size from the host plant nuclei (Fig. 1e,f), similar genome sizes were expected. To further explore the correlation between genome size and nuclear volume, and to provide further evidence that could support a genome size of *U. bidentis* close to that of *B. pilosa*, *A. sterilis* leaves infected with *P. coronata* and *C. arabica* leaves infected with *H. vastatrix* were also analysed microscopically. Although large differences in nuclear size were observed between *P. coronata* and *A. sterilis* nuclei (Fig. 1g), whose genome sizes are 244 Mbp (Tavares *et al.*, 2014) and 26 699 Mbp (Bennett and Smith, 1976), respectively, i.e. over 100 times difference in genome size, the use of *H. vastatrix* and *C. arabica* (Fig. 1h) revealed smaller differences in nuclei size, which is in accordance with the genome sizes of both the rust and host species of 797 and 2347 Mbp, respectively (Tavares *et al.*, 2014), i.e. less than three times difference.

The comparison between flow cytometric histograms of healthy and rust-infected *B. pilosa* leaves clearly enabled the identification of *U. bidentis* peaks (Fig. 2b,c), and enabled us to estimate the 1C DNA content of *U. bidentis* as 2.54 pg, corresponding to a haploid genome of 2489 ± 43 Mbp [coefficient of variation (CV), 1.7%; $n = 12$]. As noted previously for other rusts (Tavares *et al.*, 2014), 2C peaks were always observed for *U. bidentis* together with the 1C peak typical of fungal species. The nuclear DNA content of the host species, *B. pilosa*, was also estimated by comparison with the internal reference standard *S. lycopersicum* (Fig. 2a), and revealed that the *B. pilosa* genome is larger than previously thought (6.04 ± 0.15 pg/2C, i.e. 5908 Mbp vs. 3325 Mbp). This large difference in genome size is probably caused by the use of different methodologies. Although our estimation was obtained using flow cytometry, the estimation of Bennett and Leitch (1997) was obtained using Feulgen densitometry, which presents many critical procedural points (e.g. fixation, slide preparation and storage, acid hydrolysis) that determine its precision.

The genome size estimated for *U. bidentis*, 2489 Mbp, is within the range of that of the host plant, which is further corroborated by the similar nuclear sizes indicated by fluorescence microscopy observation (Fig. 1e,f). Although very large, it is interesting to note that the rust genome is smaller than the host plant genome, a situation that was recurrently observed in a study involving 32 rust–host species pairs (Tavares *et al.*, 2014). Nevertheless, the *U. bidentis* genome is approximately three times larger than the largest fungal genome reported previously (*G. confusum*, 893 Mbp), and over 50 times larger than the average fungal genome size (44.2 Mbp). Indeed, adding the genome size of *U. bidentis* to the list of 1850 fungal genomes with known size (Kullman *et al.*, 2005; Tavares *et al.*, 2014) shifts the average fungal genome size by c. 1.5 Mbp to 45.9 Mbp, the average

Basidiomycota genome size from 70.4 to 76.2 Mbp, and the average Pucciniales genome size from 305.5 to 350.9 Mbp.

This result further reinforces the unparalleled position of the Pucciniales among fungi as a group with large or very large genomes. It also stresses the huge variation in genome size occurring within the group, highlighted by the over 30 times difference between *Cronartium quercuum* f. sp. *fusiforme* Burds. & G.A. Snow (with 76.6 Mbp; Anderson *et al.*, 2010) and *U. bidentis* (at 2489 Mbp). Interestingly, genome size information available for seven species in the *Uromyces* genus ranges from 276.8 Mbp for *Uromyces rumicis* (Schumach.) G. Winter to 2489 Mbp for *U. bidentis*, illustrating important fluctuations in genome size even within a genus. Such large genome size variations within fungal genera are rare, paralleled only by the genera *Hymenoscyphus* (9–54 Mbp), *Suillus* (20–94 Mbp) and *Glomus* (14–264 Mbp), together with the rust genus *Puccinia* (77–806 Mbp) (Kullman *et al.*, 2005; Tavares *et al.*, 2014), and could suggest that variations in genome size may be an active element of fungal evolution, speciation and host specialization in some taxonomic groups. The occurrence and evolutionary significance of genome size expansion, mostly through polyploidy, and the subsequent ecological, structural and functional consequences have been shown in diverse fungi (Albertin and Marullo, 2012). Among rusts, such fluctuations in genome size are clear across their phylogeny (Tavares *et al.*, 2014). For example, *H. vastatrix*, one of the most ancestral rust lineages (Aime, 2006), has one of the largest rust genomes (797 Mbp), whereas *Puccinia malvacearum* Bertero ex Mont., basal to the Pucciniaceae (van der Merwe *et al.*, 2008), has a smaller genome (178 Mbp); nevertheless, Pucciniaceae, encompassing the genera *Puccinia* and *Uromyces*, comprises the entire genome size range among rusts. Rapid adaptive evolution in many pathogenic fungi has been shown to be linked to chromosomal rearrangements and ploidy changes (as reviewed by Croll & McDonald, 2012 and Stukenbrock and Croll, 2014), including the examples arising from *Fusarium* (Ma *et al.*, 2010) and *Mycosphaerella graminicola* (Fuckel) J. Schröt. (Goodwin *et al.*, 2011; Stukenbrock *et al.*, 2010). Intraspecific variability of genome size in fungi has been documented (Bourne *et al.*, 2014), in some cases being related to host–pathogen interactions, such as in *Geosmithia* (Veselská and Kolařík, 2015) or *Leptosphaeria maculans* Ces. & De Not. (Grandaubert *et al.*, 2014). Moreover, rust fungi with large genomes, such as *H. vastatrix*, *Phakopsora pachyrhizi* Syd. & P. Syd. and, to some extent, *P. chrysanthemi*, all rely on asexual reproduction (Tavares *et al.*, 2014). Similarly, *U. bidentis* is a hemicyclic rust (Baker, 1955), with no known aecial host. Therefore, polyploidy and/or the activity of transposable elements could be important diversity-creating factors in such mostly asexual organisms, being the main mechanisms driving genome expansions. Such relations between genome size variation and sexual abstinence and/or population size have been documented in

several organisms, including fungi (Hu *et al.*, 2014; Spanu, 2012), and have been the subject of theoretical analyses in evolutionary biology (e.g. Startek *et al.*, 2013; Whitney & Garland, 2010). With diversified reproduction strategies (sexual, asexual or, rarely, sexual), host ranges (single or few species, or several species from diverse taxa) and large but variable genome sizes, we anticipate that rust fungi may also prove to be relevant for such studies.

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