# Caracterisation of *Puccinia hemerocallidis* causing the first outbreak of daylily rust in Europe

# Caracterização de *Puccinia hemerocallidis* causadora do primeiro surto de ferrugem de lírio-de-um-dia na Europa

Short title: *Puccinia hemerocallidis* in Europe

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ABSTRACT

Daylily (*Hemerocallis* spp.) is an ornamental plant widely used in gardens. Daylily rust, caused by the fungus *Puccinia hemerocallidis*, has disseminated through all continents only in the 21th century, except in Europe, where it has been considered a quarantine disease by the European Plant Protection Organisation. In Portugal, since November 2015, typical rust symptoms were observed in daylily plants in gardens in Lisbon, Alentejo, Algarve and Madeira, attaining high prevalence, incidence and severity. The causal agent was identified as *P. hemerocallidis* and the Koch's postulates were fulfilled. Phylogenetic data suggest that this fungus may have been introduced from North America. Using flow cytometry, the genome size of the *P. hemerocallidis* populations present in Portugal was estimated to be 345 Mbp (0.3533 pg DNA/1C). For such analysis *Rhamnus alaternus* was validated as a DNA standard, exhibiting a nuclear content of 0.680 pg DNA/2C. The identification of this disease in diverse locations in Portugal represents a threat to European breeding and nursery industries, since there are the appropriate conditions for inoculum maintenance and propagation from Portugal to the rest of Europe.

Keywords: *Puccinia hemerocallidis*, *Hemerocallis*, daylily, quarantine, Europe.

RESUMO

O lírio-de-um-dia (*Hemerocallis* spp.) é uma planta ornamental muito utilizada em jardins. A ferrugem de lírio-de-um-dia, causada pelo fungo *Puccinia hemerocallidis*, disseminou-se por todos os continentes já no século 21, com exceção da Europa, onde tem sido considerada uma doença de quarentena pela Organização Europeia de Proteção das Plantas. A partir de novembro de 2015 foram observados sintomas de ferrugem em plantas de lírio-de-um-dia em jardins de Lisboa, Alentejo, Algarve e Madeira, com elevados níveis de prevalência, incidência e severidade. O agente causal foi identificado como *P. hemerocallidis*, tendo sido cumpridos os postulados de Koch. Dados filogenéticos sugerem que a introdução do fungo poderá ter ocorrido a partir da América do Norte. Através do uso da citometria de fluxo, o tamanho do genoma das populações de *P. hemerocallidis* presentes em Portugal foi estimado em 345 Mbp (0,3533 pg DNA/1C). Para tal, *Rhamnus alaternus* foi validado como padrão de DNA, exibindo uma constituição nuclear de 0,680 pg DNA/2C. A identificação desta doença em diversos locais em Portugal representa uma ameaça para os melhoradores e viveiristas europeus de lírio-de-um-dia, já que existem condições para a manutenção e propagação de inóculo a partir de Portugal para o resto da Europa.

Palavras-chave: *Puccinia hemerocallidis*, *Hemerocallis*, lírio-de-um-dia, quarentena, Europa.

Daylily (or Hemerocallis) is an ornamental plant appreciated by its long flowering period and strong chromatic coverage. It comprises the species *Hemerocallis lilioasphodelus* L. or interspecific hybrids with neighbouring species, including *H. minor* Mill., clustered in the family Xanthorrhoeaceae. The species have originated in Eastern Asia (Russia, Korea, Japan and China), but it has adapted to various climates, being cultivated from the tropics to high latitudes, with tens of thousands of varieties being present in public and private gardens throughout the world.

Daylily rust, caused by the fungus *Puccinia hemerocallidis* von Thümen, was first described in 1880 on *H. fulva* L. in Eastern Russia (von Thümen, 1880). However, it was not until the 21$^{st}$ century that the disease was reported out of Asia. In fact, in a period of only seven years the disease was reported in North America in 2000 (Williams-Woodward *et al*., 2001), in South America in 2001 (Carvalho *et al*., 2001), in Oceania in 2002 (EPPO, 2002) and in Africa in 2007 (Mostert *et al*., 2008) (Figure 1). Infected material was also detected in Europe in plants exported from the USA to the UK in 2001 and 2002, but the disease was detected on arrival and did not establish (Jones & Sansford, 2005). Thus, the European Plant Protection Organisation listed this pathogen in the A1 list of quarantine diseases (EPPO, 2015).

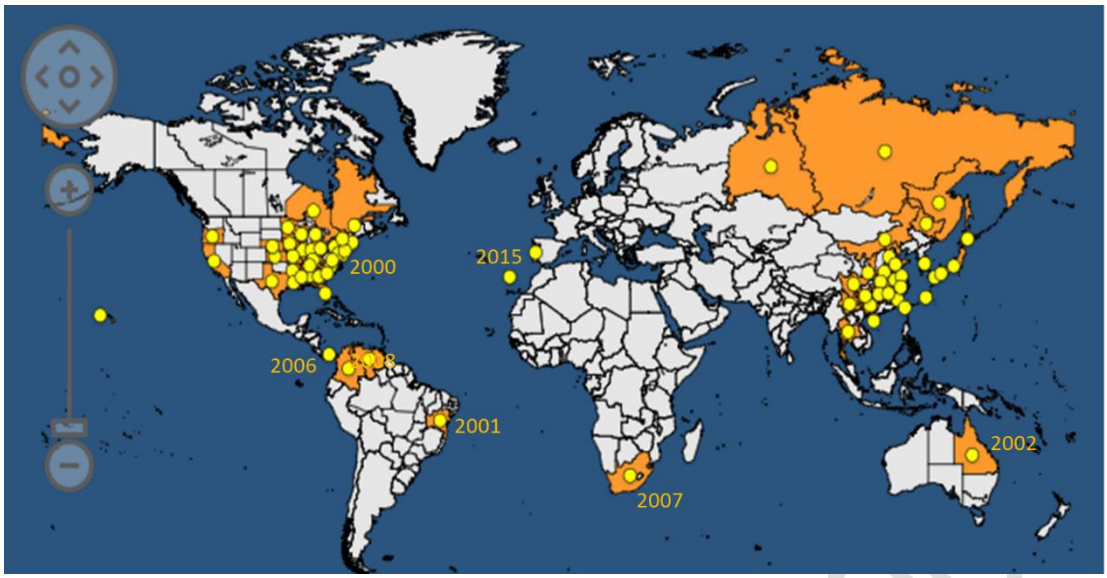


Figure 1 – Global distribution of *Puccinia hemerocallidis*, with dates of first report (adapted from EPPO Global Database, *P. hemerocallidis* distribution).

In Portugal, since November 2015, typical rust symptoms were observed in daylily plants in gardens in Lisbon, Alentejo, Algarve and Madeira, attaining 66% prevalence (the disease was present in six out of nine gardens surveyed), incidence (symptoms in 85-90% of the daylily plants) and severity (25-75% of the leaf surface covered with rust pustules) (Silva *et al*., 2016). Symptoms included small lesions originating orange/yellow urediniosporic sori on the upper page of the leaves, over which brown teleutosporic sori subsequently developed (Figure 2). Severe infections lead to defoliation and loss of vigour and of ornamental value of the plants (Figure 3). The microscopic examination of fungal structures led to the identification of *P. hemerocallidis* and the Koch's postulates were fulfilled, confirming this fungus as the causal agent of daylily rust (Silva *et al*., 2016).

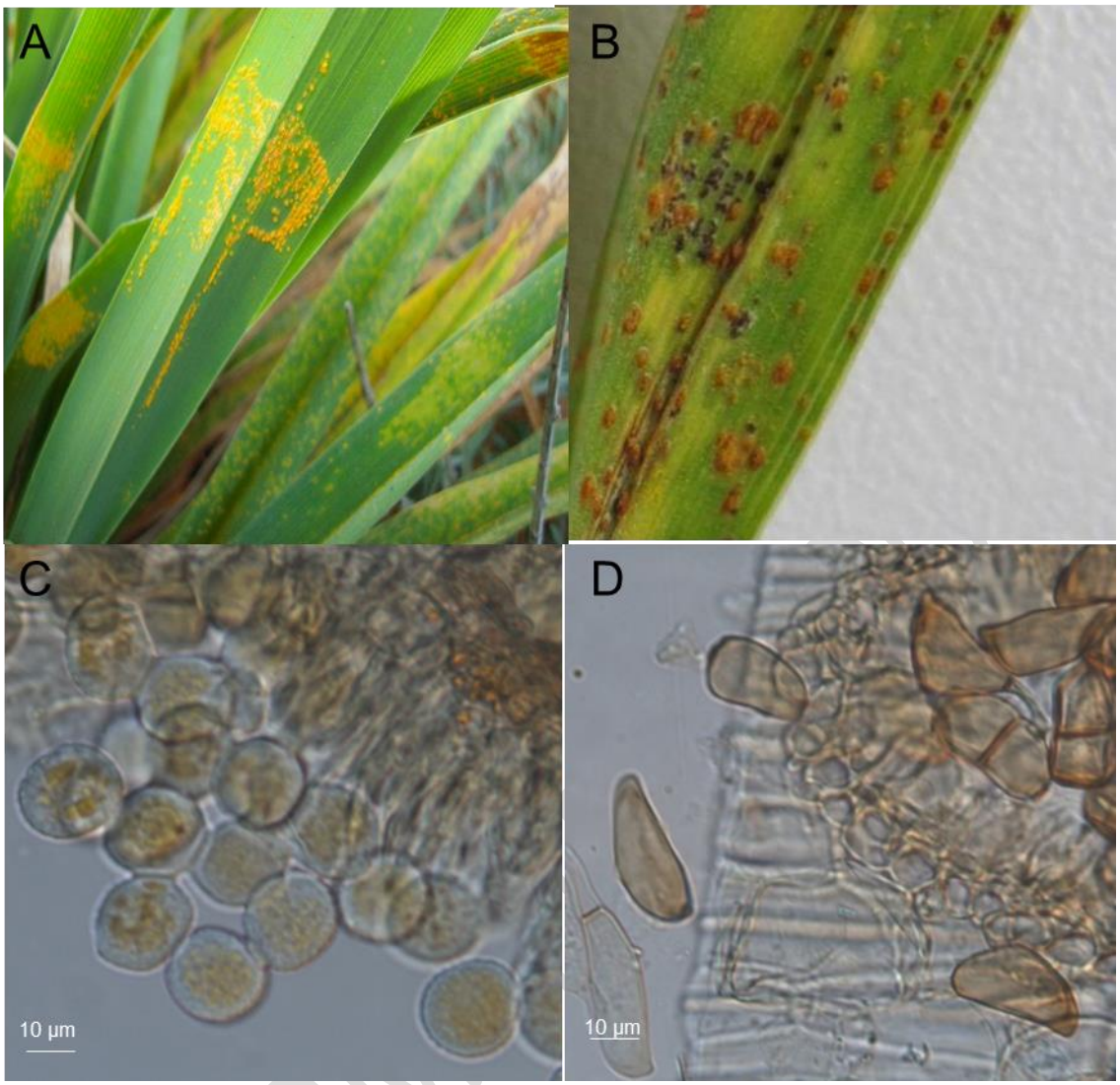


Figure 2 – *Puccinia hemerocallidis* uredinia (A and B) and telia (B), including details of urediniospores (C) and teliospores (D).

Figure 3 – Symptoms and signs of daylily rust in Porto Covo (Alentejo; left) and Tavira (Algarve; right).

Urediniospores collected from a single pustule from infected material collected at Porto Covo (Alentejo) were used for DNA extraction using a phenol:chlorophorm protocol (Talhinhas *et al*., 2003) adapted to 1.5mL tubes. The rDNA-ITS region was amplified and sequenced as previously described (Talhinhas *et al*., 2002). The nucleotide sequence (Table 1) matches entirely those of *P. hemerocallidis* from the USA and Costa Rica, while revealing to be distinct from those from other continents (Figure 4), suggesting a North- or Central-American origin of the Portuguese daylily rust outbreak. The fact that only one Portuguese sample was analysed does not enable to rule out the hypothesis of multiple introductions. On the other hand, no sequences from South American, South African or Central/Southern Asian samples are available for comparison.

Table 1 – Nucleotide sequence of the rDNA-ITS region obtained from *Puccinia hemerocallidis* DNA extracted from a single pustule derived from infected material collected at Porto Covo (Alentejo)

| region | Sequence (5'->3') |
|---|---|
| 18S ribosomal RNA | GAAGGATCATTA |
| ITS1 | AATAATCAAGAGTGCACTTTATTGTGGCTCAAAATTTACTTAATTTCACC CTGAACACTTGGTTGTGTGACTTGTTCATTGCAACCAGGTATGTGTAAC ACAATTCTTTTTTTTATTGTGTTACATTATCCCCCTCAATCATTTTTTTTT TTAAACCCAAGTTGAATTATAAGAATGTAAATATTTTAATAATAAAAAA |
| 5.8S ribosomal RNA | TAACTTTTAACAATGGATCTCTAGGCTCTCACATCGATGAAGAACACAG TGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATTGAAT CTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTG AGTGTCATGA |
| ITS2 | AAATCTCTCATCAAATTAATTTTTGGTGGATGTTGAGTGCTGCTGTTATC TAGCTCACTTTAAATATATAAGTCATTTTATGAATCTTGGATTGACTTGG TGTAATATTTTTTTGATCATCAAGGAAAGTAGCAATACTTGCCAATATTT ATTTTCAAGGACTACTAAACCCTTCAACTTATTTTTAAGA |
| 28S ribosomal RNA | CCTCAAATCAGG |

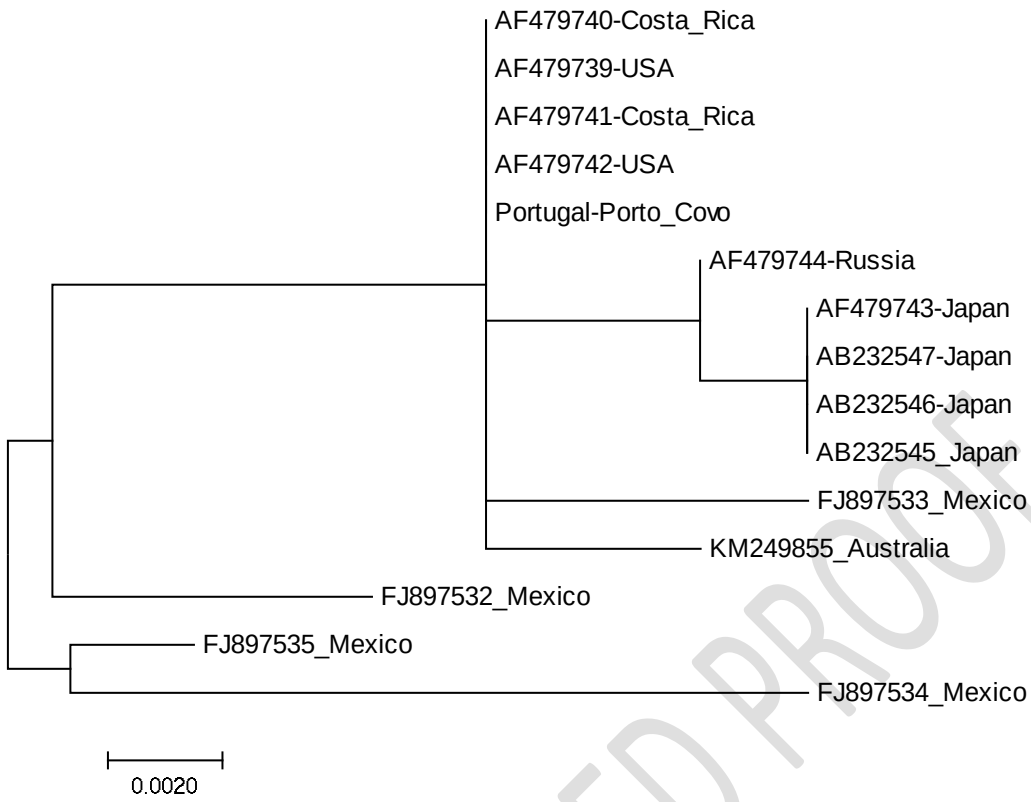


Figure 4 – Genetic diversity among *Puccinia hemerocallidis* samples collected in different continents depicted by a phylogram constructed by the Maximum Likelihood method based on the Tamura-Nei model (using the MEGA7 software under default parameters) upon ClustalW-aligned sequences retrieved from the NCBI nucleotide sequence database.

To further characterise the pathogen, and because no information was available for *P. hamerocallidis*, rust-infected leaf material was also used for genome size estimation by flow cytometry, by employing the nuclei isolation protocol developed by Loureiro *et al*. (2007) and optimized for rust fungi by Tavares *et al*. (2014). Samples were analysed using a CyFlow Space flow cytometer (Sysmex, Germany), with *Raphanus sativus* L. 'Saxa' (1.11 pg DNA/2C) and *Cenococcum geophilum* Fr. isolate 844.1 (0.208 pg DNA/1C) as reference standards (Doležel *et al*., 1998; Talhinhas *et al*., 2017. Additionally, *Rhamnus alaternus* L. (from populations naturally occurring at Tapada da Ajuda, Lisbon) was validated as DNA standard by comparison with *C. geophilum*, *Raphanus sativus* and *Solanum lycopersicum* L. 'Stupické' (1.96 pg DNA/2C; Doležel *et al*., 1992) (Figure 5), presenting a genome size of 0.680±0.028 pg DNA/2C. The genome size of *P. hemerocallidis* was thus estimated to be 345 Mbp (0.3533 ± 0.0210 pg DNA/1C) (Figure 6), a similar value to the average genome size for the Pucciniales reported by Ramos *et al*. (2015), i.e., 351 Mbp, further reinforcing the outstanding genome size of Pucciniales fungi, which are approximately seven times larger than the average fungal genome sizes (Talhinhas *et al*., 2015). The host plant was also compared to *Vicia faba* L. 'Inovec' (26.65 pg DNA/2C; Doležel *et al*., 1992), suggesting that it is diploid (9.72 pg DNA per nucleus) according to Podwyszyńska *et al*. (2015).

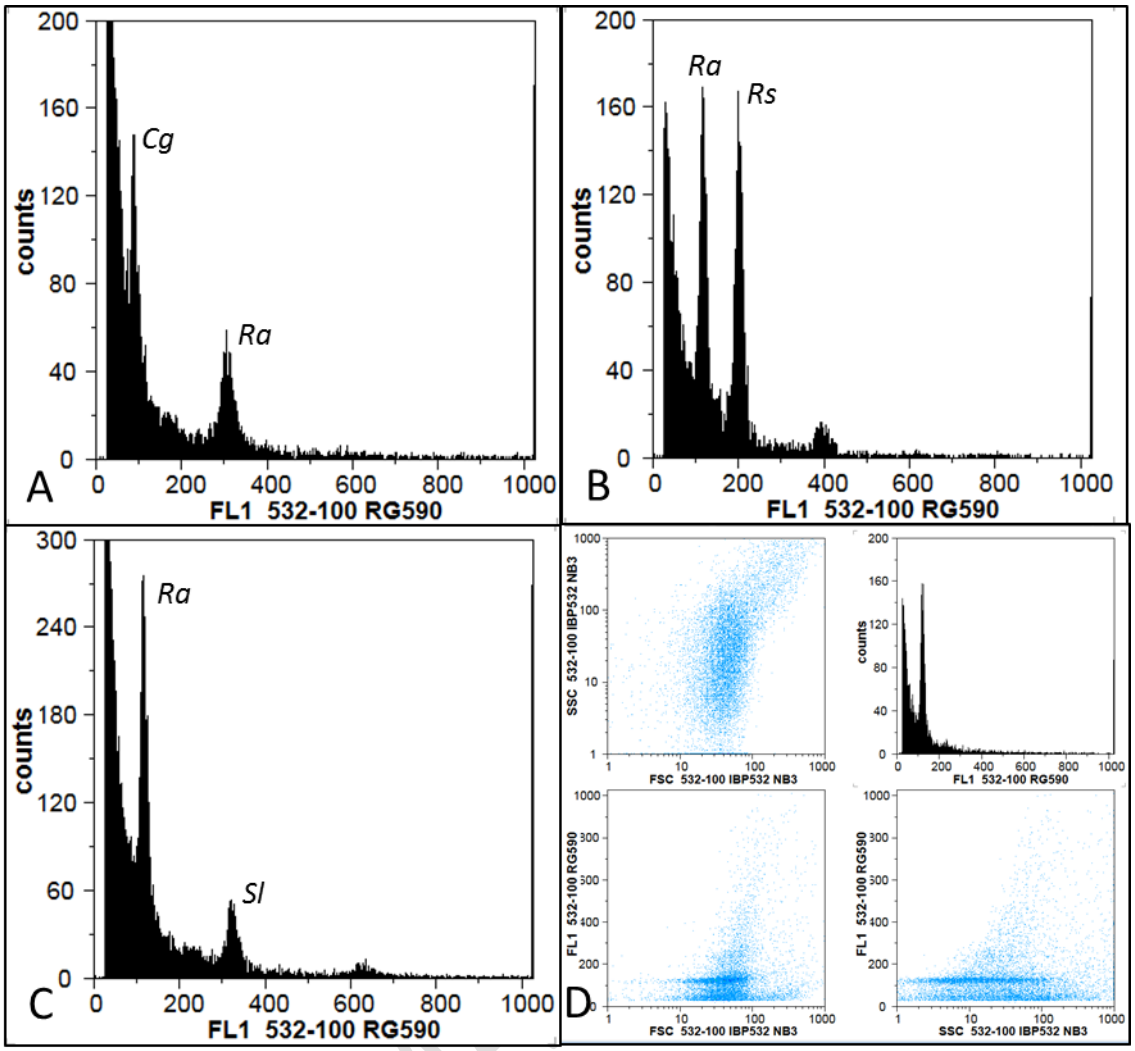


Figure 5 – Validation of *Rhamnus alaternus* (collected at Tapada da Ajuda, Lisbon) as DNA standard for genome size analysis using flow cytometry (as detailed in Figure 6), by comparison with the fungus *Cenococcum geophilum* isolate 844.1 (0.208 pg DNA/1C; panel A; Talhinhas *et al*., 2017) and the plants *Raphanus sativus* 'Saxa' (1.11 pg DNA/2C; panel B; Doležel *et al*., 1998) and *Solanum lycopersicum* 'Stupické' (1.96 pg DNA/2C; panel C; Doležel *et al*., 1992); panel D represents *Rhamnus alaternus* nuclei alone, also depicting dot-plots relating FSC, SSC and FL.

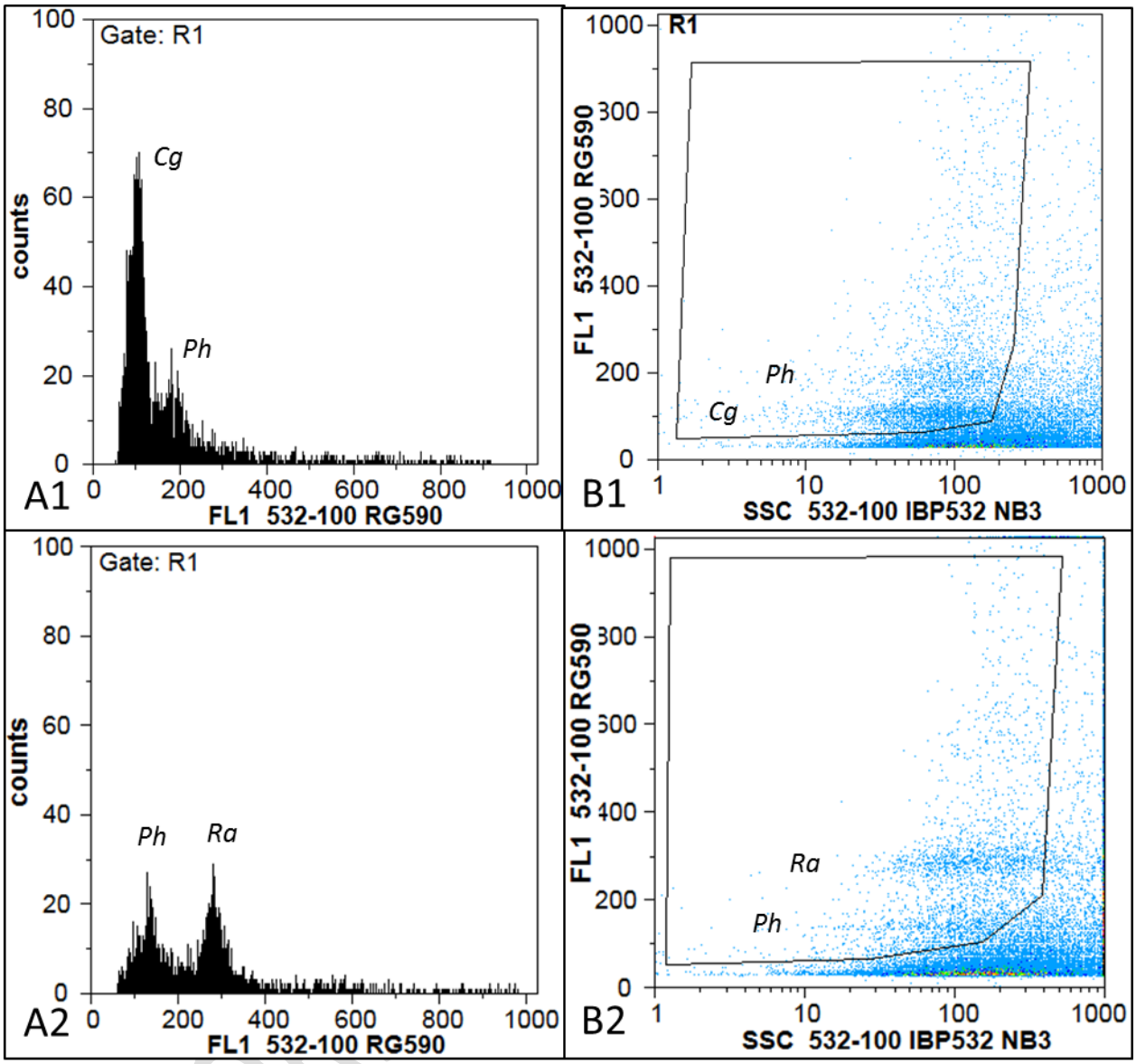


Figure 6 – Flow cytometric analyses of relative fluorescence intensities of propidium iodide-stained nuclei simultaneously isolated from *Puccinia hemerocallidis* (Ph) and the DNA reference standard *Cenococcum geophilum* (Cg, 0.208 pg DNA/1C) or *Rhamnus alaternus* (Ra, 0.680 pg DNA/2C) (panels in rows 1 and 2, respectively); panels in column A depict flow cytometric histogram of relative fluorescence intensities, while panels in column B show the gating made in the dot-plot of SSC vs. FL to exclude as much as possible partial nuclei and other types of debris.

*Puccinia hemerocallidis* is a macrocyclic heteroaecious fungus, having *Patrinia* spp. (Valerianaceae) as aecial hosts. *Patrinia* spp. do not occur out of East Asia. In cool climates daylilies lose their leaves in winter and, in the absence of the aecial host, the disease cycle is naturally broken, as urediniospores are incapable of overwintering. However, in climates with mild winter, most daylily genotypes retain their leaves, creating appropriate conditions for inoculum maintenance and survival. This contrasting scenario occurs between the North and South of the USA (Williams-Woodward *et al*., 2001) and it is likely to remain valid in contrasting climates in Europe. The identification of daylily rust in Portugal, for the first time in Europe, is therefore relevant, on one hand because the Mediterranean climate conditions in Portugal will enable inoculum survival, and on the other hand because the Western location of

this country favours inoculum dispersal East- and Northeast-wards by the dominant winds. In the USA, Mueller *et al*. (2003) and Blythe *et al*. (2015) showed that most cultivars are susceptible to the disease, although some exhibit resistance. Following the protocol by Loureiro *et al*. (2015), we have inoculated *P. hemerocallidis* urediniospores on daylily leaves collected from three different gardens in the Lisbon area (unknown cultivars) and all proved susceptible to the disease. There are over 3000 cultivars available in catalogues across Europe, many of which were European-bred, and only 10 of those were included in the study by Blythe *et al*. (2015), none being rated as resistant. It is therefore crucial that the resistance of the European cultivars is characterized, in advance of a hypothetical entry of the pathogen in other European countries. Additionally, Buck (2013) suggested the occurrence of physiological races in *P. hemerocallidis*, although neither a race differentiation system nor accompanying molecular data are available yet. Such results and the questions that remain to be answered suggest research topics that may help the management of daylily rust in Europe, along with experimental data on the biology, epidemiology and control of this pathogen (Mueller & Buck, 2003; Buck *et al*., 2010, 2011; Dong & Buck, 2011; Dong *et al*., 2013). The challenge is now on European plant pathologists and daylily breeders to join efforts to plan and deploy the most effective plant protection strategies in order to maintain the economic and aesthetical value of daylilies, while minimizing the employment of pesticides in nurseries and public gardens. While basing breeding programmes in selected resistant germplasm should provide a durable mid- to long-term disease control strategy, in the short terms all players should take extra-care when obtaining propagating material from parts of the world where the disease is present, report and destroy foci of disease and avoid the continuous presence of foliage in gardens or nurseries (thus breaking the urediniosporic cycle) namely by choosing cultivars with longer deciduous periods.

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