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INVASION NOTE

Not one but three: undetected invasive *Alnus* species in northwestern Patagonia confirmed with cpDNA and ITS sequences

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Abstract Species of *Alnus* (alders) have become invaders in several parts of the world. Here we report the presence of three naturalized alien species: *A. glutinosa*, *A. incana* and *A. rubra* from several populations in nature reserves of northwestern Patagonia, an area of remarkably high biodiversity. *Alnus glutinosa* had been cited previously for Chile and southern Argentina, but *A. incana* and *A. rubra* are here reported for the first time. As we found morphological variation within and among the populations of these introduced species that makes their discrimination difficult, we used chloroplast (*trnH-psbA*) and nuclear ribosomal (ITS) DNA sequences to confirm their identifications from morphological characteristics. Results from nuclear and chloroplast sequence data confirm the morphological tentative identification of the three species and remark the utility of molecular information together with morphology for the detection of introduced species of taxonomically difficult groups. The invasive characteristics of these alien tree species are discussed in relation to the conservation of the nature reserves where they are found.

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Introduction

Invasive trees are of special concern because they have substantial and growing impact on biodiversity, ecosystem functioning, and human lives in many regions of the world (Richardson et al. 2014). They can profoundly change environments, alter disturbance regimes, and modify water and nutrient cycles. Several Northern Hemisphere trees that have been introduced in mediterranean and temperate areas of the Southern Hemisphere have become problematic invaders. The most emblematic are species of *Pinus*, which have been reported as naturalized aliens in all continents of the Southern Hemisphere (Rejmánek and Richardson 2013). In many instances, *Pinus* species have modified both forests and treeless environments such as steppes, fynbos and high elevation vegetation (e.g., Simberloff et al. 2002). Also, species of *Salix* such as *S. fragilis* (brittle willow) have deeply altered riversides and watercourses of Argentina and New Zealand (e.g., Bogar et al. 2015), and are among the 32 weeds considered of national significance in Australia (http://www.environment.gov.au/biodiversity/invasive/weeds/weeds/lists/wons.html).

The genus *Alnus* (Betulaceae) consists of ca. 40 Northern Hemisphere species of trees and large shrubs (with one in the Northern and Central Andes), of which some have been reported as invasive in areas where they have been introduced (e.g., *A. rubra* Bong. in Australia; Csurches and Edwards 1998; *A. glutinosa* (L.) Gaertn. in North America and New Zealand; Furlow 1997, Bogar et al. 2015). Their rapid growth and nitrogen-fixing capacity, together with the small size and low mass of their abundant wind- or water-dispersed seeds, make them especially apt for invasions of wet habitats and exposed soils (Rejmánek and Richardson 1996). *Alnus glutinosa* (black alder) from Europe is cultivated as ornamental or to control erosion and improve soils in many regions of the world, and has become naturalized in North America, southern South America, New Zealand, and South Africa (Rejmánek and Richardson 2013). Recently, it was first-reported for Argentina as an exotic escaped from cultivation in the region of Nahuel Huapi National Park, and included in the catalogue of vascular plants of southern South America as the only alien *Alnus* naturalized in Chile and Argentina (Zuloaga et al. 2008).

Recent work we performed in relation to the presence of alien plants in nature reserves of northern Patagonia, Argentina, showed several *Alnus* populations distributed within two national parks of Argentina: Lanín and Nahuel Huapi, in the west of Neuquén and Río Negro provinces. These national parks comprise very diverse environments with temperate climate that include high snow-capped mountains, glacial lakes and valleys. The area is dominated by *Nothofagus*/*Austrocedrus* wet and mesic forests in the west, arid grasslands and shrub steppes in the east, and a transition ecotone between them. The region presents very high species richness, with approx.1400 plant species of the total 2400 found in Argentine Patagonia, including a large proportion of aliens (nearly 25%), mostly related to the presence of urban settlements and human activity in the region (Speziale and Ezcurra 2011). Some of these have already become problematic invaders, especially woody trees such as *Pinus contorta*, *P. ponderosa* and *Pseudotsuga menziesii* (Simberloff et al. 2010), and *Salix fragilis* (Bogar et al. 2015).

The *Alnus* plants we found in these reserves presented morphological variation within and among populations, which suggested the existence of other species in addition to *A. glutinosa* in the area. As *Alnus* is a relatively large and taxonomically complex genus with ca. 40 species from the Northern Hemisphere (Li and Skvortsov 1999), to identify the naturalized alien species in this region we combined morphological and molecular analyses. A molecular approach such as barcoding is especially relevant in cases of taxonomically difficult, species-rich and widely distributed genera, where morphological regional identification keys cover only fractions of the species total number. Plant barcodes are typically used in an integrative fashion with other information for detecting species. Sometimes, unexpected sequence divergence has led to re-examination of morphological variation, and in others, morphological variants have led to generating sequence data for recognizing different taxa (Hollingsworth et al. 2016). Also, barcoding using a combination of DNA markers from different genomes, such as nuclear and plastid which have different modes of inheritance and track different evolutionary histories, can further our understanding of species delimitation and the evolutionary processes related to speciation (Li et al. 2011).

Methods and results

Fourteen naturalized populations of *Alnus* have been detected within Lanín and Nahuel Huapi National Parks in northwestern Patagonia, Argentina (Edwards 2011). Most of them were found on coasts of lakes, but some were associated to borders of watercourses. Among them all, six populations were chosen as to represent the extent of distribution and the morphological variation found among populations (Fig. 1). One to three specimens were collected in each, depending on the amount of intrapopulation morphological variability observed (Table 1). They were identified tentatively using morphological characters of keys and descriptions published in *Flora Europaea* (Tutin et al. 1964), *Flora of North America* (Furlow 1997) and *Flora of China* (Li and Skvortsov 1999) as *A. glutinosa*, *A. incana* (L.) Moench and *A. rubra* (Table 2).

Total genomic DNA was obtained from 20 mg of dried leaf tissue using the Purelink Plant Total DNA Purification Kit (Invitrogen, Carlsbad, CA, USA). The chloroplast (cp) *trnH-psbA* and the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS)

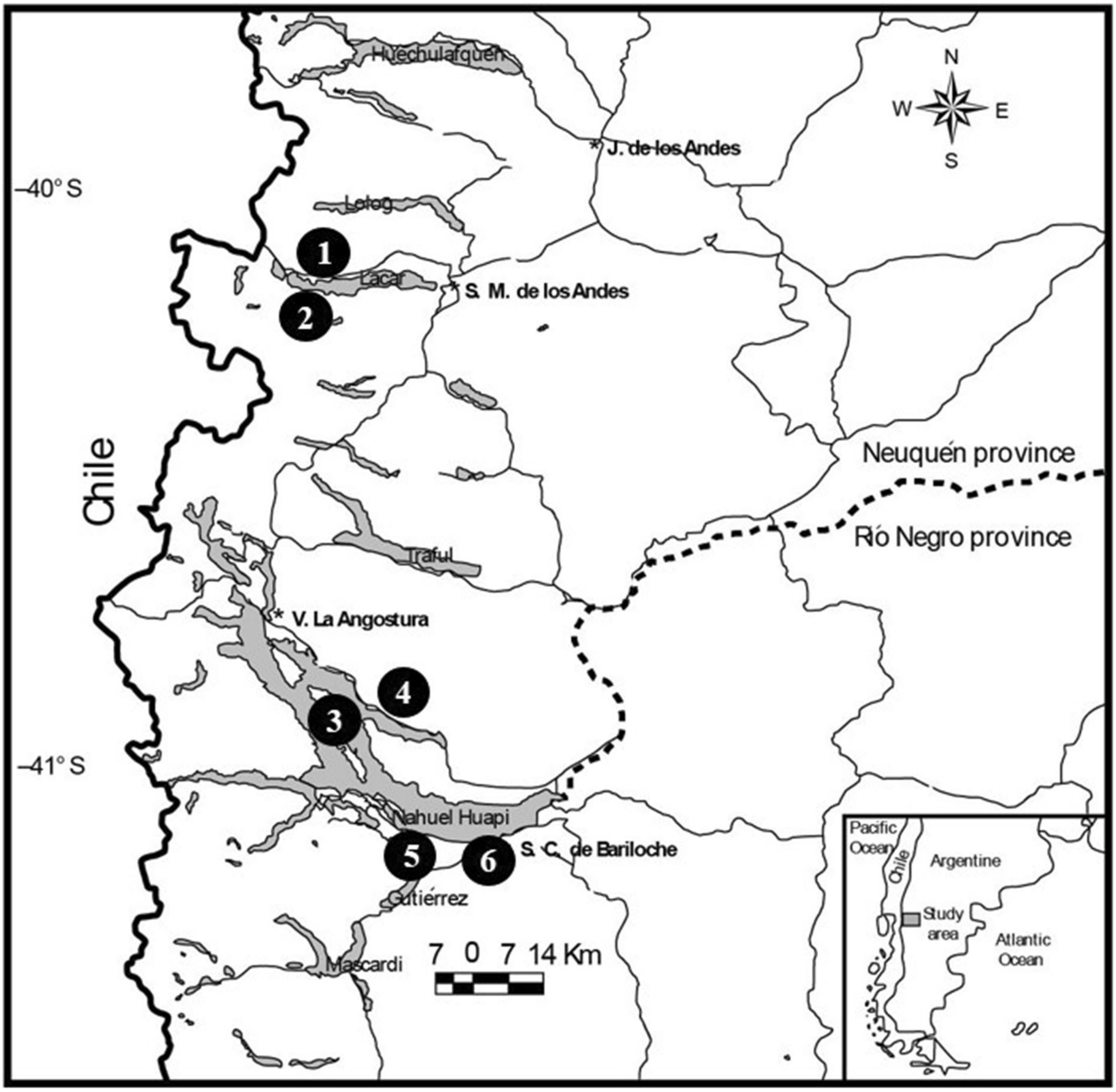


Fig. 1 Map of Northwestern Patagonia, Argentina, with analyzed populations of *Alnus* species marked as black circles. The populations 1 (Quechuquina—40°09′42.8″S, 71°34′22.0″W) and 2 (Ruca Ñire—40°10′45.8″S; 71°34′35.3″W) are located in Lanín National Park. The populations 3 (Isla Victoria, Playa del Toro—40°58′34″S; 71°31′25.9″W), 4 (Santa María—40°56′23.74″S; 71°23′32.93″W), 5 (Lago Gutiérrez—41°09′46.79″S; 71°24′24.11″W) and 6 (Pinar de Festa—41°07′03.79″S; 71°24′07.11″W) are located in Nahuel Huapi National Park

regions were PCR-amplified using universal primers and standard methods (Ren et al. 2010). All sequencing was done using an ABI (Applied Biosystems, Carlsbad, CA, USA) 3730XL high-throughput DNA capillary sequencer at Macrogen (Seoul, Korea). Newly obtained sequences (Table 1) were compared against the nucleotide database of GenBank using their standard nucleotide blast alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nuclear ITS sequences from the Patagonian accessions matched perfectly (100% identity) with GenBank accessions with the same identifications: tentatively identified Patagonian *A. incana* with two GenBank *A. incana* (FJ825401.1; AJ251665.1),

Table 1 New accessions of *Alnus* (Betulaceae) from which nrDNA ITS and cpDNA *trnH-psbA* sequences were obtained

Taxon	DNA accession no.	Voucher information	GenBank no. (*trnH-psbA*; ITS)
Alnus glutinosa	CC-366	Argentina. Prov. Neuquén. PN Lanín, Lago Lácar, Estancia Quechuquina. May-2010. *Edwards P. 105* (BCRU)	MF692799; MF692808
Alnus glutinosa	CC-419	Argentina. Prov. Neuquén. PN Nahuel Huapi. Cerca de Santa María. 02-feb-2012. *Ezcurra C. 3762* (BCRU)	MF692801; MF692809
Alnus glutinosa	CC-422	Argentina. Prov. Neuquén. PN Nahuel Huapi. Cerca de Santa María. 02-feb-2012. *Ezcurra C. 3764* (BCRU)	MF692802; MF692810
Alnus glutinosa	CC-430	Argentina. Prov. Río Negro. Bariloche. Pinar de Festa, cultivada. 02-feb-2012. *Ezcurra C. 3760* (BCRU)	MF692803; MF692811
Alnus glutinosa	CC-431	Argentina. Prov. Neuquén. PN Lanín. Lago Lácar. Ruca Ñire. May-2010. *Edwards P. 101* (BCRU)	MF692800; MF692812
Alnus glutinosa	CC-432	Argentina. Prov. Neuquén. Nahuel Huapi. Isla Victoria. Playa del Toro. Mar-2010. *Edwards P. 102* (BCRU)	MF692804; MF692813
Alnus incana	CC-406	Argentina. Prov. Río Negro. PN Nahuel Huapi. Lago Gutierrez entre El Retorno y Arelauquen. 02-feb-2012. *Calviño C.I. 805* (BCRU)	MF692796; MF692805
Alnus incana	CC-420	Argentina. Prov. Neuquén. PN Nahuel Huapi. Cerca de Santa María. 02-feb-2012. *Ezcurra C. 3763* (BCRU)	MF692797; MF692806
Alnus incana	CC-421	Argentina. Prov. Río Negro. Bariloche. Pinar de Festa. 02-feb-2012. *Ezcurra C. 3761* (BCRU)	MF692798; MF692807
Alnus rubra	CC-405	Argentina. Prov. Neuquén. Nahuel Huapi. Isla Victoria. Playa del Toro. Mar-2010. *Edwards P. 104* (BCRU)	MF692795; MF692814

Table 2 Morphological characteristics of the accessions of *Alnus* from Patagonia tentatively identified as *A. glutinosa*, *A. incana* and *A. rubra*. The DNA accession numbers correspond to those in Table 1

Taxon	*Alnus glutinosa*	*Alnus incana*	*Alnus rubra*
DNA accession no.	CC-: 366, 419[a], 422, 430, 431, 432	CC-: 406, 420, 421	CC-405
Leaf shape	Obovate to orbicular	Narrowly ovate to widely elliptic or orbicular	Ovate to elliptic
Leaf apex	Generally rounded or retuse	Obtuse, acute or acuminate	Obtuse or acute
Leaf margin	Flat, doubly serrate	Flat, doubly serrate	Markedly revolute, doubly serrate to crenate
Number of secondary veins	5–8 pairs	7–12 pairs	9–14 pairs
Abaxial leaf surface	Green and glabrous, but with hairs in the axiles of lateral veins, frequently resinous	Grayish-tomentose, not resinous	Pale green, glabrous or very scarcely pubescent, not resinous
Peduncle length of pistillate inflorescences	>5–20 mm	1–5 mm	1–10 mm

[a]Displays morphological intermediacy with *A. incana* in leaf surface

Patagonian *A. glutinosa* with five GenBank *A. glutinosa* (FJ825399.1, AY352310.1, AJ251662.1, FJ825403.1, FJ825404.1), and Patagonian *A. rubra* with two GenBank *A. rubra* and one *A. rugosa* (FJ825402.1, AJ251668.1, AJ251667.1). Chloroplast *trnH-psbA* sequences from the Patagonian accessions

Table 3 Sequence characteristics of the cpDNA *trnH-psbA* and nrDNA ITS regions for 117 and 71 accessions of Betulaceae, respectively

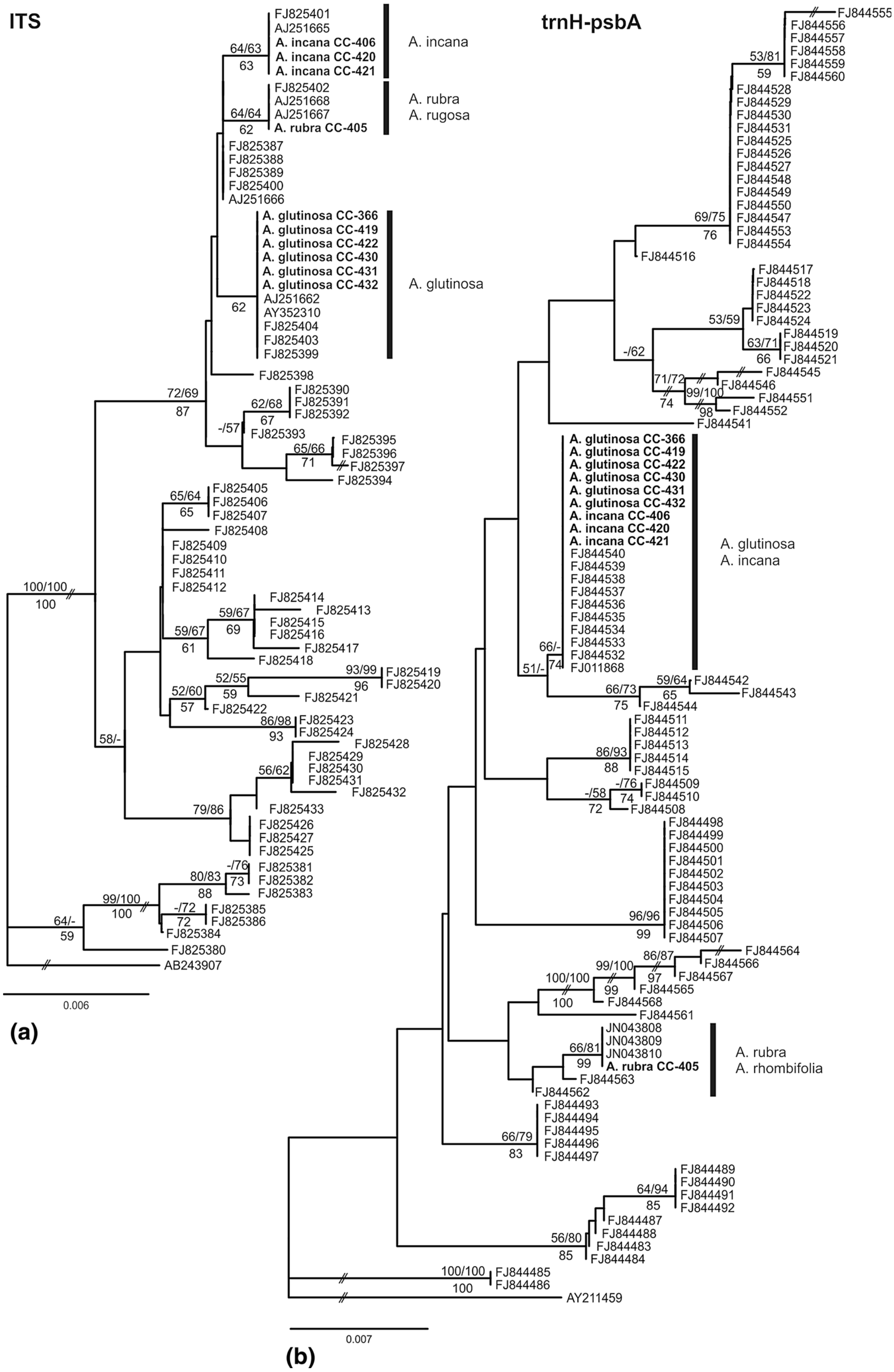
Sequence characteristic	*trnH-psbA*	ITS
Length variation (range)		
Alnus	297–422	517–527
Alnus glutinosa	362–371	526
Alnus incana	371	526
Alnus rubra	408	526
No. aligned positions	463	528
No. positions eliminated	19	0
No. positions not variable	362	452
No. positions autapomorphic	22	33
No. positions parsimony informative	60	43
No. unambiguous alignment gaps	14	5
Sequence divergence (range)		
All taxa included	0–10.63	0–8.77
Alnus glutinosa versus *Alnus incana*	0	0.38
Alnus glutinosa versus *Alnus rubra*	1.38	0.53
Alnus incana versus *Alnus rubra*	1.38	0.49

of *A. glutinosa* and *A. incana* had 100% match to two GenBank accessions of *A. glutinosa* (FN687522.1, FJ011868.1), while the Patagonian *A. rubra* perfectly matched three GenBank accessions identified as *A. rubra* and *A. rhombifolia* (JN043810.1, JN043809.1, JN043808.1).

All ITS and *trnH-psbA* sequences from a study on DNA barcoding of *Alnus* (Ren et al. 2010) plus additional sequences from phylogenetic studies of the genus (Navarro et al. 2003; Chen and Li 2004) were downloaded from GenBank and two datasets were constructed, including the Patagonian accessions; sequence and alignment characteristics are indicated in Table 3. These datasets were used to estimate neighbor-joining (NJ) trees in PAUP* v.4.0b10 (Swofford 2002) to evaluate the accessions based on their sequence similarity. Maximum parsimony, NJ, and maximum likelihood bootstrap values were calculated from 1000 replicates using heuristic and NJ search strategies in PAUP* v.4.0b10 (Swofford 2002), and the rapid bootstrap algorithm of RAxML (Stamatakis 2006), respectively. The ITS NJ tree grouped the six Patagonian accessions of *A. glutinosa* with other five GenBank accessions of the same species; likewise the three Patagonian *A. incana* were grouped with two GenBank accessions of *A. incana*, and the Patagonian *A. rubra* fell with two GenBank accessions of *A. rubra* and an identical *A. rugosa* (Fig. 2a). In the *trnH-psbA* NJ tree the Patagonian *A. glutinosa* and *A. incana* clustered together with other GenBank accessions of the same two species, and the Patagonian *A. rubra* fell together with GenBank accessions of *A. rubra* and *A. rhombifolia* (Fig. 2b). Even though our accessions of *A. rubra* also grouped with *A. rugosa* (ITS NJ tree) and *A. rhombifolia (trnH-psbA* NJ tree), morphological characteristics of the latter two species allowed us to conclude that the Patagonian accessions correspond to *A. rubra*. The leaf blade margins of *A. rugosa* are flat, whereas the Patagonian accessions have strongly revolute margins, as *A. rubra* (Furlow 1997; Table 2). Similarly, the leaf margins of *A. rhombifolia* are serrulate or finely serrate, without noticeably larger secondary teeth, whereas the Patagonian accessions are doubly serrate or crenate, with distinctly larger secondary teeth, as *A. rubra* (Furlow 1997; Table 2).

Discussion

Nuclear ITS and cpDNA *trnH-psbA* sequences confirm the morphological tentative identification of the specimens as belonging to three alien species, *Alnus glutinosa*, *A. incana*, and *A. rubra*. The ITS NJ tree clearly places the accessions from the populations of northwestern Patagonia identified as such in the same three groups as GenBank accessions of each of these species from the Northern Hemisphere (Eurasian *A. glutinosa* and *A. incana*, and North American *A. rubra*). The cpDNA tree produces comparable results, as accessions identified as *A. rubra* from Patagonia are most similar to those of Northern Hemisphere *A. rubra*, and those identified as *A. incana* and *A. glutinosa* group with those of these last two taxa. Even though the latter are not discriminated in different groups in the cpDNA tree, they are clearly separated from *A. rubra* and the rest of *Alnus* species included in the analysis. Therefore, this work reports the presence of naturalized *A. rubra* and *A. incana* in northwestern Patagonia, a region markedly recognized for its high biodiversity, for the first time, and confirms the occurrence of *A. glutinosa* in the region based on molecular results. Indeed, although never widely cultivated, the three species were introduced for cultivation in experimental stations of Argentina's


ITS
FJ825401
AJ251665
A. incana CC-406
A. incana CC-420
A. incana CC-421
64/63
63
A. incana
FJ825402
AJ251668
AJ251667
A. rubra CC-405
64/64
62
A. rubra
A. rugosa
FJ825387
FJ825388
FJ825389
FJ825400
AJ251666
A. glutinosa CC-366
A. glutinosa CC-419
A. glutinosa CC-422
A. glutinosa CC-430
A. glutinosa CC-431
A. glutinosa CC-432
AJ251662
AY352310
FJ825404
FJ825403
FJ825399
62
A. glutinosa
FJ825398
72/69
87
FJ825390
FJ825391
FJ825392
62/68
67
-/57
FJ825393
FJ825395
FJ825396
FJ825397
65/66
71
FJ825394
FJ825405
FJ825406
FJ825407
65/64
65
FJ825408
FJ825409
FJ825410
FJ825411
FJ825412
100/100
100
FJ825414
FJ825413
59/67
69
FJ825415
FJ825416
FJ825417
59/67
61
FJ825418
93/99
96
FJ825419
FJ825420
52/55
59
FJ825421
52/60
57
FJ825422
86/98
93
FJ825423
FJ825424
58/-
FJ825428
FJ825429
FJ825430
FJ825431
56/62
FJ825432
79/86
FJ825433
FJ825426
FJ825427
FJ825425
-/76
73
FJ825381
FJ825382
FJ825383
80/83
88
99/100
100
-/72
72
FJ825385
FJ825386
FJ825384
64/-
59
FJ825380
AB243907
0.006
(a)
trnH-psbA
FJ844555
FJ844556
FJ844557
FJ844558
FJ844559
FJ844560
53/81
59
FJ844528
FJ844529
FJ844530
FJ844531
FJ844525
FJ844526
FJ844527
FJ844548
FJ844549
FJ844550
FJ844547
FJ844553
FJ844554
69/75
76
FJ844516
FJ844517
FJ844518
FJ844522
FJ844523
FJ844524
53/59
FJ844519
FJ844520
FJ844521
63/71
66
-/62
FJ844545
71/72
FJ844546
99/100
FJ844551
74
98
FJ844552
FJ844541
A. glutinosa CC-366
A. glutinosa CC-419
A. glutinosa CC-422
A. glutinosa CC-430
A. glutinosa CC-431
A. glutinosa CC-432
A. incana CC-406
A. incana CC-420
A. incana CC-421
FJ844540
FJ844539
FJ844538
FJ844537
FJ844536
FJ844535
FJ844534
FJ844533
FJ844532
FJ011868
A. glutinosa
A. incana
66/-
74
51/-
59/64
FJ844542
66/73
65
FJ844543
75
FJ844544
FJ844511
FJ844512
FJ844513
FJ844514
FJ844515
86/93
88
-/76
FJ844509
-/58
74
FJ844510
72
FJ844508
FJ844498
FJ844499
FJ844500
FJ844501
FJ844502
FJ844503
FJ844504
FJ844505
FJ844506
FJ844507
96/96
99
FJ844564
86/87
FJ844566
99/100
FJ844567
100/100
97
99
FJ844565
100
FJ844568
FJ844561
JN043808
JN043809
JN043810
66/81
A. rubra CC-405
99
FJ844563
A. rubra
A. rhombifolia
FJ844562
FJ844493
FJ844494
FJ844495
FJ844496
FJ844497
66/79
83
FJ844489
FJ844490
FJ844491
FJ844492
64/94
85
FJ844487
FJ844488
56/80
FJ844483
85
FJ844484
100/100
FJ844485
FJ844486
100
AY211459
0.007
(b)

◀**Fig. 2** Neighbour-joining trees of **a** nrDNA ITS and **b** cpDNA *trnH-psbA* DNA sequences showing genetic similarity between invasive *Alnus* species from Patagonia, Argentina (in bold), and accessions of species available in GenBank. MP and ML bootstrap support values are shown above branches (left/right, respectively), and NJ bootstrap values below branches

National Parks during the middle of last century (Koutché 1942). The descriptions and key differences between these naturalized alien species have been included in *Flora Argentina* (Ezcurra and Calviño 2017).

It is interesting to note that in some places such as near Santa María we found morphologically diverse populations of *A. glutinosa* and *A. incana* growing together and displaying morphological intermediates between the two species (e.g. DNA accession CC-419, Tables 1 and 2). This specimen however was unequivocally clustered with one of the species based on ITS, but the two species showed no molecular differentiation based on cpDNA *trnH-psbA* sequence data (Table 3). This scenario can be indicative of either a lower mutation rate in plastid DNA compared with ITS for these two species, or possible hybridization and introgression leading to chloroplast capture, as similarly discussed by Li et al. (2011). Previous studies have suggested hybridizations between *A. glutinosa* and *A. incana* (e.g., Banaev and Bažant 2007). Future population genetic studies in these variable populations could unravel these possibilities.

The analyses presented here show the utility of molecular information for the detection of introduced species of taxonomically difficult groups. Indeed, the three *Alnus* species reported, *A. glutinosa*, *A. rubra* and *A. incana,* belong to a circumpolar species-complex of closely related species, probably due to a recent history of diversification (Chen and Li 2004). Populations of this complex in different biogeographic areas have been recognized as either different species, or subspecies of the same species (Chen and Li 2004). The morphological variation within the species of the *A. incana* complex and the minimal differences between them makes their identification problematic. Therefore, in taxonomically difficult cases such as this, the use of molecular information from different genomes, in addition to morphology, can be essential.

Also, this study highlights the utility of molecular tools for the early identification of invasive trees. Diagnostic characters of tree species are often in reproductive structures that sometimes appear only after several years or even decades of vegetative growth. In vegetative stage, these species are sometimes difficult to identify morphologically, so the use of molecular information in the context of a complete established database of species sequence data can be an important strategy for their early detection and intervention in relation to the protection and management of native ecosystems.

Although reported as invasive at other sites (e.g., *A. rubra* in Australia, Csurches and Edwards 1998; *A. glutinosa* in North America and New Zealand, Furlow 1997, Bogar et al. 2015), the species of *Alnus* occurring in northwestern Patagonia are considered naturalized, but not invasive to date (Edwards 2011). Even so, these species exhibit many of the typical intrinsic characteristics of invasive species, such as early sexual maturation, small seed size, abundant seed production, abiotic seed dispersal, and vegetative reproduction capacity (Rejmánek and Richardson 1996, Sakai et al. 2001). In addition, *Alnus* is a nitrogen-fixing species that can change nitrogen levels in nutrient-poor soils, and thus, as was observed in other invasive species, promote its own invasion or that of other introduced species (Gaertner et al. 2014). Moreover, potential competition with native woody nitrogen-fixing species (e.g., *Ochetophila trinervis, Colletia hystrix, Discaria chacaye, D. articulata*) could occur since, like *Alnus*, they form actinorhizal symbiosis with *Frankia*, a genus of soil actinomycetes (Chaia et al. 2010).

On the other hand, the native ecosystems of northwestern Patagonia where *Alnus* species grow have several characteristics that may favor the invasion process (e.g. Wiser and Allen 2006). This region presents numerous lakes connected by rivers and streams providing many riparian habitats, which are being widely modified by tourism, logging and grazing by domestic and introduced wild ungulates (Speziale and Ezcurra 2011). These disturbances may increase habitat susceptibility to the dispersion of *Alnus* by generating adequate establishment microsites, as these species are good colonizers of recently cleared or unstable substrates (Furlow 1997). These factors (intrinsic species traits, habitat characteristics, and disturbances) highlight the importance of developing a regular monitoring program to detect an abrupt increase of number or ranges of *Alnus* populations in order to prevent possible invasions in the highly biodiverse conservation areas of northwestern Patagonia.

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