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## Towards a universal barcode of oomycetes – a comparison of the *cox1* and *cox2* loci

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### Abstract

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Y.J.C. and M.T. conceived the study and designed the experiments; GB, S.G., J.K., H.D.S., R.G.S., H.V. and Y.J.C. provided materials; Y.J.C. conducted experiments with contributions from B.N., L.N., S.P. and S.T.; Y.J.C. and M.T. analysed and interpreted the data; and YJC and M.T. wrote the manuscript with contributions from G.B., S.G., H.D.S., R.G.S. and H.V.

#### Data accessibility

DNA sequences: GenBank Accession nos KJ654007-KJ654304 & KP684521-KP684912 (in Table S1). Sequence alignments and phylogenetic trees: TreeBase Study Accession no. 16949; URL: <http://purl.org/phylo/tree-base/phyloids/study/TB2:S16949>.

Oomycetes are a diverse group of eukaryotes in terrestrial, limnic and marine habitats worldwide and include several devastating plant pathogens, for example *Phytophthora infestans* (potato late blight). The cytochrome c oxidase subunit 2 gene (*cox2*) has been widely used for identification, taxonomy and phylogeny of various oomycete groups. However, recently the *cox1* gene was proposed as a DNA barcode marker instead, together with ITS rDNA. The *cox1* locus has been used in some studies of *Pythium* and *Phytophthora*, but has rarely been used for other oomycetes, as amplification success of *cox1* varies with different lineages and sample ages. To determine which out of *cox1* or *cox2* is best suited as a universal oomycete barcode, we compared these two genes in terms of (i) PCR efficiency for 31 representative genera, as well as for historic herbarium specimens, and (ii) sequence polymorphism, intra- and interspecific divergence. The primer sets for *cox2* successfully amplified all oomycete genera tested, while *cox1* failed to amplify three genera. In addition, *cox2* exhibited higher PCR efficiency for historic herbarium specimens, providing easier access to barcoding-type material. Sequence data for several historic type specimens exist for *cox2*, but there are none for *cox1*. In addition, *cox2* yielded higher species identification success, with higher interspecific and lower intraspecific divergences than *cox1*. Therefore, *cox2* is suggested as a partner DNA barcode along with ITS rDNA instead of *cox1*. The *cox2*-1 spacer could be a useful marker below species level. Improved protocols and universal primers are presented for all genes to facilitate future barcoding efforts.

## Keywords

barcoding; cytochrome oxidase; herbarium specimen; mtDNA; oomycete-specific primers

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## Introduction

Oomycetes belong to the kingdom Straminipila, a remarkably diverse group that includes brown algae and planktonic diatoms, although they have previously been classified under the kingdom Fungi with which they share several convergent traits, such as mycelial growth and osmotrophic nutrition. These organisms have evolved both saprophytic and pathogenic lifestyles, and more than 60% of the known species are pathogens on plants (Thines & Kamoun 2010). The majority of these plant pathogens are classified into the order Peronosporales (includes downy mildews, *Phytophthora* and *Pythium*), which cause important diseases of crops and ornamental plants, as well as forest trees. Recent phylogenetic investigations have revealed that the diversity of oomycetes has been largely underestimated. However, our knowledge of oomycete diversity is largely biased to the economically relevant plant pathogens, and little is known about saprobic or marine oomycetes (Hulvey *et al.* 2010; Nigrelli & Thines 2013). Consequently, even if all of the approximately 2000 published names were valid, the number of oomycetes is still only about one-tenth of the expected species number (Hawksworth 2001). Although morphology is the most valuable criterion for their identification and diversity, morphological species identification is time-consuming and in some groups very difficult, especially for nontaxonomists. DNA barcoding is a fast and reliable tool for the identification of species, enabling us to unravel the diversity and distribution of oomycetes.

Two regions, the internal transcribed spacer (ITS) rDNA and cytochrome oxidase (*cox*) 1 mtDNA, were recently suggested as standard DNA barcode markers for oomycetes (Robideau *et al.* 2011). However, there are several problems that arise from the choice of these loci. The use of the ITS rDNA gene is problematic for two reasons. First, for more than ten genera including the economically important genera *Bremia* and *Plasmopara* of the Peronosporales, the order with the highest diversity in Oomycetes, ITS-based studies are almost completely lacking. This is largely due to extremely long ITS sizes of up to more than 3000 bp, caused by long repetitive insertions, which make it difficult to amplify and sequence these loci (Thines 2007c). Second, there are certain cases where the ITS region exhibits insufficient variability for phylogenetic distinction in closely related species, for example in *Phytophthora* (Goodwin *et al.* 1999; Cooke *et al.* 2000; Jung & Burgess 2009) and *Peronospora* (Choi *et al.* 2007b; Voglmayr *et al.* 2014b). Due to these limitations of the ITS region, additional genes, *cox1* and *cox2*, have been used as phylogenetic markers within oomycetes (Hudspeth *et al.* 2000, 2003; Cook *et al.* 2001). The *cox1* locus has been used primarily in studies of *Pythium* and *Phytophthora* (de Cock & Levesque 2004; Kroon *et al.* 2004; Bala *et al.* 2010; Robideau *et al.* 2011; Schroeder *et al.* 2013), while the *cox2* locus has been widely used in phylogenetic studies in the downy mildews (Göker *et al.* 2007; Thines 2007b, 2011; Thines *et al.* 2007, 2008, 2009b, 2010; Choi *et al.* 2009a,c, 2011c,d; Hulvey *et al.* 2010; Runge *et al.* 2011; Schröder *et al.* 2011; Telle *et al.* 2011; Telle & Thines 2012; Thines & Kummer 2013; Testen *et al.* 2014) and also in *Pythium* (Martin 2000; Villa *et al.* 2006; Senda *et al.* 2009; Uzuhashi *et al.* 2010), *Phytophthora* (Martin & Tooley 2003a,b; Villa *et al.* 2006), white blister rusts (Choi *et al.* 2006, 2007c, 2008, 2009b, 2011a,e; Thines *et al.* 2009a; Ploch *et al.* 2010, 2011; Choi & Thines 2011; Mirzaee *et al.* 2013) and basal oomycetes (Cook *et al.* 2001; Sekimoto *et al.* 2007; Beakes & Sekimoto 2009; Hulvey *et al.* 2010). The success of *cox1* amplification has been variable in obligate plant pathogenic downy mildews, and as a result, the *cox2* gene has been more widely used for molecular identification at fine taxonomical levels such as within a species complex (Choi *et al.* 2006, 2007c, 2008, 2009a, 2011a,d; Schröder *et al.* 2011; Thines & Kummer 2013) and also for population genetic studies (Bowers *et al.* 2007; Choi *et al.* 2011c; Runge *et al.* 2011; Quesada-Ocampo *et al.* 2012). With the recent development of DNA extraction and PCR amplification, the *cox2* locus often successfully amplified from dried herbarium specimens over a hundred years old (Telle & Thines 2008; Choi & Thines 2011). Considering the widespread use of the *cox2* gene, its ease of amplification and its good performance on historic specimens, the question arises as to whether this is a better DNA barcode gene than *cox1* for oomycetes.

It is thus the aim of the present work to compare the utility of the *cox1* and *cox2* loci as DNA barcode markers for 31 representative genera of oomycetes, including some from old herbarium specimens. In addition, the *cox2-1* spacer that sits between *cox1* and *cox2* is analysed and compared with these two genes. We describe new methods to improve PCR success with newly developed oomycete-specific primers in conjunction with previously published primers.

## Materials and methods

### Oomycete sampling

A total of 31 genera were selected to ensure representative coverage of many oomycetes, including all major lineages of downy mildews and other Peronosporales such as *Pythium* and *Phytophthora*, Albuginales, Haptoglossales, lagenidiaceous oomycetes and Saprolegniales. Obligate biotrophic oomycetes were collected or loaned from international herbaria, while culturable oomycetes were sampled, isolated and cultivated as described by Nigrelli & Thines (2013). Three hundred herbarium specimens including 110 species of *Peronospora* (one specimen for each species) and 33 species of *Bremia* (several specimens for each species) were also sequenced and analysed to compare species identification success, nucleotide diversity and overall mean intra- and interspecific divergence. In addition, 24 herbarium specimens of *Albugo candida* and 12 of *Plasmopara viticola*, collected between 1876 and 2003, were selected to test the PCR efficiency on old herbarium samples, which are often the only source of rare species and could be used to link a species to a sequence from its type. In this study, we regard *Albugo lepigoni* and *A. ipomoeae-aquaticae* as belonging to two distinct genus-level lineages that differ from other genera of the Albuginaceae, based on both phylogenetic inference and morphology (Voglmayr & Riethmüller 2006; Y.J. Choi & M. Thines, unpublished data). The collection information for each sample, including geographic origin, collection source and date, is listed in Table S1 (Supporting information).

### DNA extraction

For obligate plant pathogens that cannot be grown in artificial media, 5–20 mg of infected plant tissue were taken from dried herbarium specimens, and disrupted in a mixer mill (MM2, Retsch, Hann, Germany), using three iron beads of 3 mm diam. per sample. From herbarium specimens collected before the year 1990, genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytic Jena AG, Jena, Germany) with PTB (Telle & Thines 2008), while for younger herbarium specimens, the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) was used on a Kingfisher Flex (Thermo Scientific, Dreieich, Germany). For culturable samples, genomic DNA was extracted from 0.25 cm<sup>2</sup> agar plugs of pure cultures using a protocol (Nigrelli & Thines 2013) modified from May & Ristaino (2004). DNA of *Haptoglossa* and *Myzocytiopsis* was extracted as described by May & Ristaino (2004).

### PCR amplification and sequencing

Four mitochondrial regions, *cox1*, *cox2*, *cox2-1* spacer and a region spanning *cox2* and *cox2-1* spacer, were amplified by PCR using nine combinations of eleven primers (Fig. 1; Tables 1 and 2): primers OomCox1-levup and OomCox1-levlo or FM85mod (Robideau *et al.* 2011) for the *cox1* region, Cox2-F and Cox2-R (Hudspeth *et al.* 2000) or Cox2-RC4 (designed here) for *cox2*, FM79-C1 (designed here) and FM80 (Martin & Tooley 2003a), FMPhy-10b (Martin *et al.* 2004) or OomCoxS-R2 (designed here) for *cox2-1* spacer, Cox2-F and FMPhy-10b or OomCoxS-R3 (designed here) for a region spanning the *cox2* and *cox2-1* spacer. Amplification reactions were carried out in 25  $\mu$ L with genomic DNA from 1 to 3 ng, 1x Mango PCR Buffer, 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub>, 0.8 mg/mL BSA, 0.4  $\mu$ m forward and

reverse primers, and 0.5 Unit Mango Taq Polymerase (Bioline GmbH, Luckenwalde, Germany). PCR conditions were as follows: an initial denaturation step of 95 °C for 4 min; 36 cycles of 95 °C for 40 s, primer set-specific annealing temperature for 40 s (see Table 2) and 72 °C extension for 60 s; and final extension of 5 min at 72 °C. For a spanning region of *cox2* and *cox2*-1 spacer, the annealing time was extended to 60 s. For all primer sets, annealing temperatures were optimized through gradient PCR. To compare the effects of the annealing temperature on PCR success for *cox1* and *cox2* genes, amplifications were performed at different temperatures. All PCR products were evaluated for successful amplification using gel electrophoresis (8 V/cm) on a 1.5% agarose gel for 1 h, with reference to a DNA marker, HyperLadder I (Bioline GmbH). PCR products were directly sequenced with the primers identical to those used for amplifications using the ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with the following modifications: a reaction volume of 20  $\mu$ L, with 0.0625 $\times$  ready reaction premix, 1 $\times$  BigDye sequencing Buffer, 3.2 pmol primer, 2–5 ng of PCR template and ddH<sub>2</sub>O. Cycle sequencing was as follows: an initial denaturation step of 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 50 °C for 40 s and 60 °C for 4 min. The sequencing products were purified using the BigDye Xterminator Purification Kit (Applied Biosystems). Amplicon sequencing was performed on the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) at the DNA sequencing laboratory of the Biodiversity and Climate Research Centre (BiK-F). All sequences were blasted at <http://ncbi.nlm.nih.gov/blast/Blast.cgi>, using the megablast algorithm and default search parameters (Altschul *et al.* 1990) to confirm their identity. GenBank Accession numbers are listed in Table S1 (Supporting information).

### Evaluation of sequence polymorphism and distance

Sequences were edited using the DNASTAR computer package (DNASTar, Inc., Madison, Wis., USA), version 5.05. An alignment of *cox1*, *cox2* and *cox2*-1 spacer sequences for three data sets, representing Oomycetes, *Peronospora* and *Bremia* (Table S1, Supporting information), was performed using MAFFT 7 (Kato & Standley 2013). DNASP (Librado & Rozas 2009) was used to calculate DNA polymorphism statistics for each loci. Interspecific (between species pairs) sequence divergence was calculated by uncorrected 'p' (percentage) distance model using MEGA 5.0 (Tamura *et al.* 2011). Minimum evolution analysis was used to construct the phylogenetic tree in MEGA 5.0, with the Tamura-Nei model. The reproducibility of the internal branches from the resulting trees was tested by bootstrap analysis using 1000 replications. Species identification, intra- and interspecific distance analyses for the *Bremia* data set were carried out using TaxonDNA/SpeciesIdentifier 1.7.8 (Meier *et al.* 2006). The frequency of intra- and interspecific uncorrected 'p' distances was plotted to check whether intraspecific and interspecific variation were overlapping. Species identification success was evaluated using two different criteria, Best Match (BM) and Best Close Match (BCM), as outlined by Meier *et al.* (2006). The BM criterion assigns a species name to the query sequence based on its best barcode match. The identification is considered 'success' when the query sequence and the matching one are from the same species, mismatched names are considered 'misidentified', and equally good matches from different species are considered 'ambiguous'. The BCM is similar to BM except that the relative frequency of all intraspecific distances is plotted to determine a threshold calculated from pairwise metrics

for each marker. All sequences without a match below the threshold value remain 'no match'.

### Primer design

To design oomycete-specific primers, alignments were generated using CLUSTALX (Thompson *et al.* 1997). Using PRIMERSELECT software (DNASTAR, Inc.), the new primers, Cox2-RC4, FM79-C1, OomCoxS-R2 and OomCoxS-R3 (Table 2), were newly designed or modified from previously published ones, to optimize them for successful amplification for all oomycete genera sequenced. The primer Cox-RC4 was modified from Cox2-R (Hudspeth *et al.* 2000), by considering a few potentially critical nucleotide differences found throughout different oomycete genera, which are described in Appendix S1 (Supporting information). Primer oligonucleotides were ordered from Sigma-Aldrich (Taufkirchen, Germany).

## Results

### Comparison of PCR success at different annealing temperatures

Previously, two different annealing temperatures were used for PCR amplification of *cox1* (55 °C; Robideau *et al.* (2011)) and *cox2* (50 °C; Hudspeth *et al.* (2000)). In this study, better PCR performance was achieved at 50 °C, for each two primer sets of *cox1* and *cox2* (Table 2). This optimal annealing temperature for both the *cox1* and *cox2* primer sets in this study was also confirmed through gradient PCR; the amplification performance gradually decreased with increase in annealing temperature from 50 to 55 °C. The barcoding primer set of *cox1*, OomCox1-levup and FM85mod failed to amplify most samples at 55 °C, excluding a few genera with a faint band, although the success was slightly improved at 50 °C. As no unspecific band was observed even at 50 °C, we used this annealing temperature on subsequent experiments for both genes. For *cox2*-1 spacer and a region spanning *cox2* and the *cox2*-1 spacer, optimal annealing temperatures were 52 and 54 °C, respectively (Table 2).

### Evaluation of primers

Nine combinations of 11 primers (Table 2), including four new oomycete-specific primers, were compared for PCR success and efficiency. For *cox1*, the reverse primer OomCox1-levlo considerably increased the average PCR success rate, compared with FM85mod, regardless of the annealing temperature, although the resulting amplicon of 680 bp was slightly smaller than 727 bp produced with FM85mod. The primer set, OomCox1-levup and FM85mod, may not be useful for herbarium specimens with relatively low DNA content, compared to DNA extracted from cultures.

For *cox2*, two different reverse primers Cox2-R and Cox2-RC4, together with the forward primer Cox2-F, successfully amplified the region for all samples and genera tested. Amplification with the new primer Cox-RC4 was more robust than with Cox2-R and generated higher amounts of amplified product, in particular for *Bremia*, *Graminivora* and *Pustula*. While the nucleotide sequence divergence between the two primers is minimal, there are a few potentially critical nucleotide differences that may explain the better performance of the Cox2-RC4 primer (see Appendix S1, Supporting information).

For the *cox2*-1 spacer region, three different reverse primers, FM80, FMPhy-10b and OomCoxS-R2, were tested together with the forward primer FM79-C1. The primer FM80 failed to amplify 12 of 31 samples, while the latter two primers successfully amplified the region in all samples. Although the latter two primers showed similar PCR success and efficiency, we prefer to use the FMPhy-10b, as the resulting sequences were 47 bp longer than OomCoxS-R2 and overlapped partially with the sequences resulting from *cox1* primer set. Primers for *cox2*-1 spacer often produced a faint, nonspecific band below the target amplicon, which did not affect sequencing. To amplify a region spanning *cox2* and the *cox2*-1 spacer, two reverse primers, OomCoxS-R3 and FMPhy-10b, were used together with the forward primer Cox2-F. Both primers led to successful amplification of all of the oomycete lineages (Fig. 2), but FMPhy-10b generated lower amounts of amplified product for *Albugo lepigoni*, *Pustula*, *Wilsoniana* and *Graminivora* than OomCoxS-R3.

The newly developed primers, Cox2-RC4 (targeting *cox2* gene), OomCoxS-R2 (*cox2*-1 spacer), OomCoxS-R3 (*cox2* and *cox2*-1 spacer) and FM79-C1 (*cox2*-1 spacer), are oomycete specific. Although we extracted the DNA from infected plant tissue that may have also contained plant, insect, fungal or bacterial DNA, no co-amplification of nontarget DNA was observed. The newly developed primers improved the overall results when compared with previously published primers with respect to amplification performance (data not shown).

### Cox1 vs. *cox2* as a barcoding locus

The primer set specific for *cox2* successfully amplified all oomycete genera included in our study, while the primer sets for *cox1* did not amplify *Eraphthora*, *Sclerophthora* and *Albugo lepigoni* (Fig. 2). The PCR failure of the three lineages by the *cox1* primer sets was confirmed by a further study including four *Eraphthora*, five *Sclerophthora*, one *A. lepigoni* and ten *A. occidentalis* samples (a species closely related with *A. lepigoni*) (data not shown). This indicated that the *cox1* primer sets are not useful for at least these groups. The *cox1* primer set amplified a single fragment of 680 bp in the other oomycete samples tested. The size of *cox2* products is slightly different, mostly 581 bp, but 572 bp in *Pachymetra* and 593 bp in Albuginales with the exception of *A. lepigoni* (599 bp).

For historic herbarium specimens of *A. candida* and *Plasmopara viticola*, the utility of *cox1* and *cox2* genes was compared with regard to amplification and sequencing. The *cox2* primers successfully amplified all herbarium specimens regardless of the specimen ages of *A. candida* and generated higher amounts of amplified product than with *cox1* primers (Fig. 3). However, *cox1* could not be amplified from four samples (collected in 1983, 1966, 1951 and 1887) and showed only a faint band for several 100-year-old samples. The PCR success for a spanning region of *cox2* and *cox2*-1 spacer decreased with the ages of the specimens. Interestingly, all primer sets resulted in a bright band for the oldest herbarium specimen collected in 1876. To confirm whether recent contamination was the source of amplification as might be expected from old material with supposedly low DNA concentration (Andreasen *et al.* 2009), the sequence of the amplicon was determined and compared with other sequences of *A. candida*. The resulting sequences were identical to a previously published sequence of *A. candida* from *Berteroa incana*, with the same two nucleotide positions that

are different from other *Albugo candida* sequences. This indicated that the amplicon originated from the herbarium specimen and not from contamination. Compared to *A. candida*, PCR amplification was less robust for the herbarium specimens of *P. viticola*. However, the *cox2* primers successfully amplified 11 of the 12 up to 100-year-old specimens, but the primer sets for *cox1* amplified only two samples collected very recently, in 1997 and 2003.

The nucleotide diversity and the interspecific (between species) variation of *cox1* and *cox2* genes for three data sets, representing 26 Oomycete genera, 110 *Peronospora* and 33 *Bremia* species, represented with one sample, are presented in Table 3. For these three data sets, *cox2* gene presents consistently higher nucleotide diversity than *cox1*:  $0.15563 \pm 0.000405$  for Oomycete genera,  $0.0959 \pm 0.013$  for *Peronospora* species and  $0.09696 \pm 0.03804$  for *Bremia* species in *cox2* gene, while in *cox1*  $0.13067 \pm 0.0000122$ ,  $0.08581 \pm 0.010$  and  $0.09668 \pm 0.03641$ , respectively (*t*-test:  $P < 0.001$  in Oomycete and *Peronospora*, but  $P > 0.05$  in *Bremia* data sets). For *Peronospora* species, *cox2* yielded a slightly higher level of species identification success of 98.2% (108 of 110 species), than *cox1* of 96.4% (106 of 110 species). This difference is due to a discriminatory power for four related species of *Peronospora* parasitic on Caryophyllaceae, *P. scleranthi*, *P. honckenya*, *P. parva* and *P. cerastii-brachypetali*. In the *cox1* gene, these cannot be distinguished from one another. In contrast, the *cox2* gene discriminated them into three genotypes (Fig. S1, Supporting information). For the *Bremia* data set, *cox2* successfully discriminated all species, but *cox1* failed to distinguish one species. Mean pairwise distances of *cox1* and *cox2* varied with the three data sets, but *cox2* always presented higher interspecific divergences of 0.1586, 0.0877 and 0.0890, respectively, than *cox1* with 0.1331, 0.0786 and 0.0881 (Table 3, Fig. 4).

For 190 samples representing 33 species of the *Bremia*, intraspecific (within species) and interspecific (between species) distances for each marker were calculated and plotted in Fig. 5. The mean intraspecific variations of all markers were low, ranging from 0.0011 (*cox2*, *cox2*-1 spacer) to 0.0017 (*cox1*), and the mean interspecific variation was higher for the *cox2*-1 spacer than *cox1* and *cox2*. The maximum intra- and interspecific variations of *cox2*-1 spacer were much higher than *cox2* and *cox1*. As the minimum interspecific variation was zero for both *cox1* and *cox2*-1 spacer and very low (0.0019) for *cox2*, intra- and interspecific distances overlapped for all markers. However, the *cox2* represents the least observations for interval overlap (3.59% of all observations), while the *cox1* and *cox2*-1 spacer showed a higher percentage of overlap observations, 11.49% and 13.75%, respectively. At 90% overlap, excluding the largest 5% of the intraspecific and the lowest 5% of the interspecific distances, however, no overlap was found for any of the markers. Species identification results using the two criteria, Best Match (BM) and Best Close Match (BCM), are displayed in Fig. 6 and Table S2 (Supporting information). Both BM and BCM criteria showed similar results for the three markers. However, the best identification success was obtained for the *cox2* (96.31% for BM and 94.73% for BCM), followed by the *cox1* (93.68% for BM and 92.63% for BCM), while the *cox2*-1 spacer obtained least success (78.42% for BM and 70.00% for BCM) with much higher score of no match (10.52%).



## Cox2-1 spacer

The region spanning *cox2* and the *cox2-1* spacer is diverse in the size of the amplicons; in two genera, *Saprolegnia* and *Pachymetra*, it was much smaller (789 bp and 825 bp, respectively) than in other genera, in which it ranged from 911 bp (*Basidiophora*) to 985 bp (*A. lepigoni*). The variation observed between the genera is mostly due to length differences in the *cox2-1* spacer. The primer set for the *cox2-1* spacer region resulted in fragments smaller than 420 bp., which were highly polymorphic in both nucleotide differences and lengths (Table 3). The insertion of repeat sequences, which were commonly found in this region, requires many gaps during the process of alignment, leading to poor alignments, especially for different oomycete genera. In addition, despite higher nucleotide diversity and interspecific divergence for all data sets (Table 3, Figs 4 and 5), identification success of *cox2-1* spacer was much lower than *cox1* and *cox2* (Table S2, Fig. 6).

## Discussion

Numerous oomycete species are pathogenic to many different host plants, and while most obligate biotrophic pathogens are highly host specific, the hemibiotrophic and necrotrophic species have broader host ranges (Beakes & Sekimoto 2009; Thines & Kamoun 2010; Lara & Belbahri 2011; Beakes *et al.* 2012). Due to the ability to spread rapidly to new areas or countries, especially by global trade with seeds and plants, but also by ballast water, pathogenic oomycetes pose a significant threat for horticulture, forestry, agriculture and aquaculture (Brasier 2008; Grunwald *et al.* 2012). Their prompt detection and precise identification are essential prerequisites for implementation of proper control strategies. DNA barcode markers will improve the success rate of identification of such potential pathogens. For the oomycetes, some loci have been useful for barcoding, for example the nuclear ribosomal internal transcribed spacer (ITS) and the mitochondrial loci cytochrome c oxidase 1 (*cox1*) and 2 (*cox2*) genes. However, the applicability of these loci has not yet been tested systematically. As the ITS region has large insertions for some oomycete species (exceeding 2500 bp), which can affect both ITS1 (Voglmayr 2003; García-Blázquez *et al.* 2008) and ITS2 (Thines *et al.* 2005; Choi *et al.* 2007a; Thines 2007a), this locus is not suitable as a general barcode. When the ITS rDNA and *cox1* mtDNA genes were suggested as barcode markers (Robideau *et al.* 2011), no comparison was made to the performance of the *cox2* mtDNA locus, which has widely been used for phylogenetic studies at the species level and has shown a high resolution (Martin 2000; Cook *et al.* 2001; Hudspeth *et al.* 2003; Martin & Tooley 2003a,b; Choi *et al.* 2006, 2007c, 2008, 2009a,b,c, 2011a,c,d,e; Villa *et al.* 2006; Göker *et al.* 2007; Sekimoto *et al.* 2007; Thines 2007b, 2011; Thines *et al.* 2007, 2008, 2009a,b, 2010; Beakes & Sekimoto 2009; Senda *et al.* 2009; Hulvey *et al.* 2010; Ploch *et al.* 2010, 2011; Uzuhashi *et al.* 2010; Choi & Thines 2011; Runge *et al.* 2011; Schröder *et al.* 2011; Telle *et al.* 2011; Telle & Thines 2012; Mirzaee *et al.* 2013; Thines & Kummer 2013; Testen *et al.* 2014; Voglmayr *et al.* 2014a,b). For the largest group of oomycetes, the obligate biotrophic plant pathogens (downy mildews and white blister rusts), *cox1* has only rarely been used (40 species with this locus in GenBank in Dec. 2014), but a larger database is already present for *cox2* (170 species with this locus in GenBank in Dec. 2014). For studies of early diverging oomycetes, *cox2* has been proven to be a suitable locus (Cook *et*

*al.* 2001; Hakariya *et al.* 2007, 2009; Sekimoto *et al.* 2007, 2008; Hulvey *et al.* 2010), whereas there are only a few *cox1* sequences of some of these available.

By analysing 31 representative oomycete genera and old historic herbarium specimens, we determined that the *cox2* sequence yielded better PCR performance than *cox1*. While 3 of 31 representative oomycete lineages were not showing satisfactory amplification for *cox1*, all 31 lineages could be amplified for the *cox2* locus. This is most likely due to highly conserved regions that enable stable anchoring of oligonucleotide primers, which seems to be more variable for *cox1* as the two reverse primers of *cox1* were not conserved throughout some oomycete lineages. Although we designed several new primers for conserved regions of *cox1*, none of them could improve the PCR performance (data now shown). In addition, *cox2* was more effective in identifying the largest genus of Oomycete, *Peronospora*, at the species level than *cox1*, due to its higher nucleotide diversity and interspecific divergence. Our study also showed that a region covering the *cox2* gene, together with the hyper-variable *cox2*-1 spacer, can be amplified from all oomycete lineages tested with a newly developed primer, highlighting its potential as a powerful tool for identification, population genetics and phylogenetic studies in a variety of oomycetes. Due to interspecific sequence variation, the *cox2*-1 spacer was previously considered useful for the development of species-specific markers and sequence-based identification of *Phytophthora* species (Martin & Tooley 2003a; Martin *et al.* 2004; Grunwald *et al.* 2011). The availability of the spacer region for a large panel of oomycetes will allow future intraspecific genetic studies that may provide much needed insights into host plant specialization, colonization history and biogeography of these plant pathogens.

For the past three centuries, thousands of oomycete specimens have been deposited in 3400 internationally recognized herbaria around the world (Thiers 2014). These specimens are an invaluable resource, especially for the study of obligate plant pathogens, which are unculturable in artificial media, for example white blister rust and downy mildew pathogens. This is not only because many species are rare and not easily collected, but also because the name of a species is ultimately linked to its type specimen. Thus, for the purpose of species identification by barcoding, it is also advantageous if historic type specimens are accessible along with barcoding primers. Although it is probably more complicated to amplify a target gene by PCR from DNA extracted from historic herbarium specimens than to sequence and map its whole genome (Yoshida *et al.* 2013) because of DNA degradation and fragmentation, there are many cases in which the *cox2* sequence has value. Notably, *cox2* may be used for accurate species identification and phylogenetic studies of herbarium specimens, especially in the two oomycete groups, Albuginales (Choi *et al.* 2007c, 2008, 2009b, 2011a,e; Thines *et al.* 2009a; Ploch *et al.* 2010; Choi & Thines 2011) and Peronosporales (Thines *et al.* 2008, 2009b, 2010; Hulvey *et al.* 2010; Choi *et al.* 2011d; Runge *et al.* 2011; Schröder *et al.* 2011; Thines 2011; Telle & Thines 2012). These studies include several cases of extracting DNA from specimens from the 19th century (Choi *et al.* 2007c, 2011a,c; Telle & Thines 2008; Thines *et al.* 2009a; Runge *et al.* 2011) and type specimens from the 19th century and the first half of the 20th century (Choi *et al.* 2009b,c, 2011b,e). In the present study, the *cox2* primer set successfully amplified old herbarium collections, including four specimens collected in the late 19th century, an intensive period of research of oomycete systematics, while *cox1* could not be amplified from most of these

using the same conditions. In the case of the *P. viticola* specimens tested, *cox1* could not be amplified from any specimen older than 16 years. Even if amplification failed for the full *cox2* region of old specimens, it may be possible to obtain a smaller fragment in semi-nested PCRs, as Telle & Thines (2008) have shown that this approach often yields amplification from DNA from which the complete fragment could not be obtained. However, this approach is not available for the *cox1* locus. In conclusion, our results suggest we should reconsider the usefulness of *cox1* as a universal barcode for oomycetes and we provide evidence that *cox2* is better suited to this because of its ease of amplification among oomycete lineages, better performance on herbarium specimens, higher discriminatory power at the species level and the availability of a large taxonomically diverse database that already includes many species of oomycetes, especially the downy mildews. A *cox2*-based identification system will be more effective in understanding the biodiversity and distribution of oomycetes. The increased availability of historic and sometimes well-referenced herbarium specimens should facilitate species identification and resolve taxonomic issues, as well as provide insights into evolutionary questions through a comparative analysis between extant and historic pathogens.

## Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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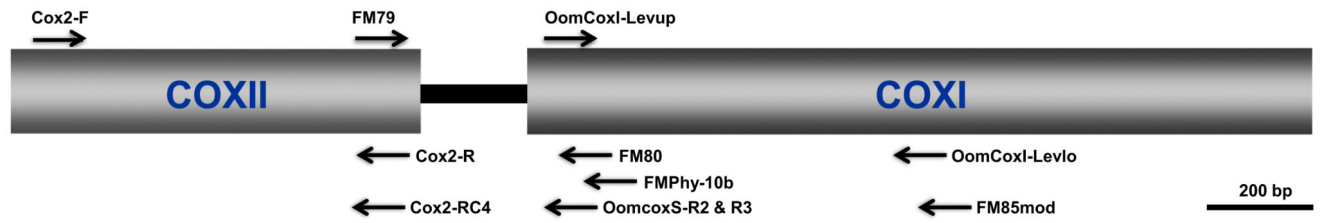
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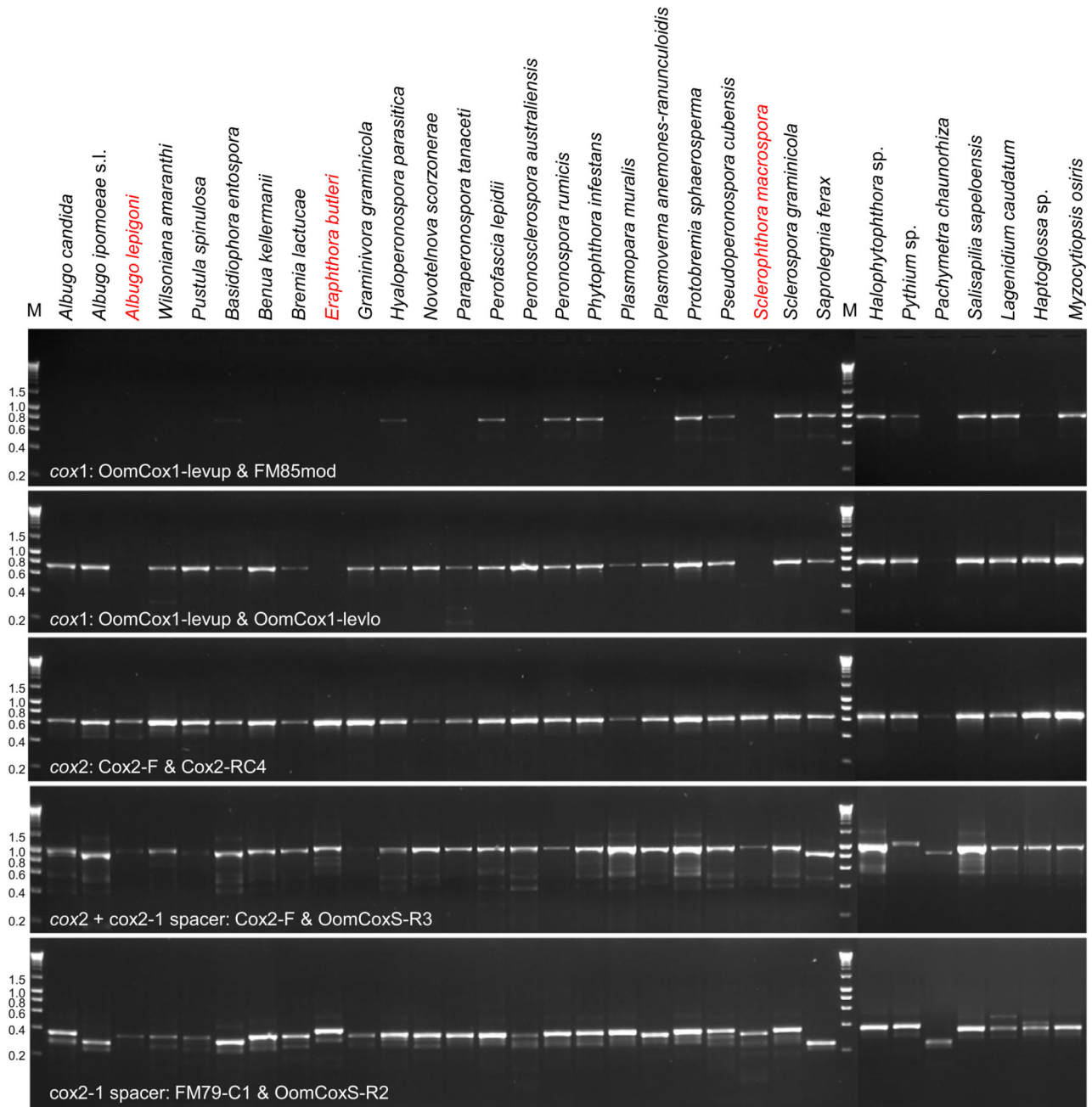
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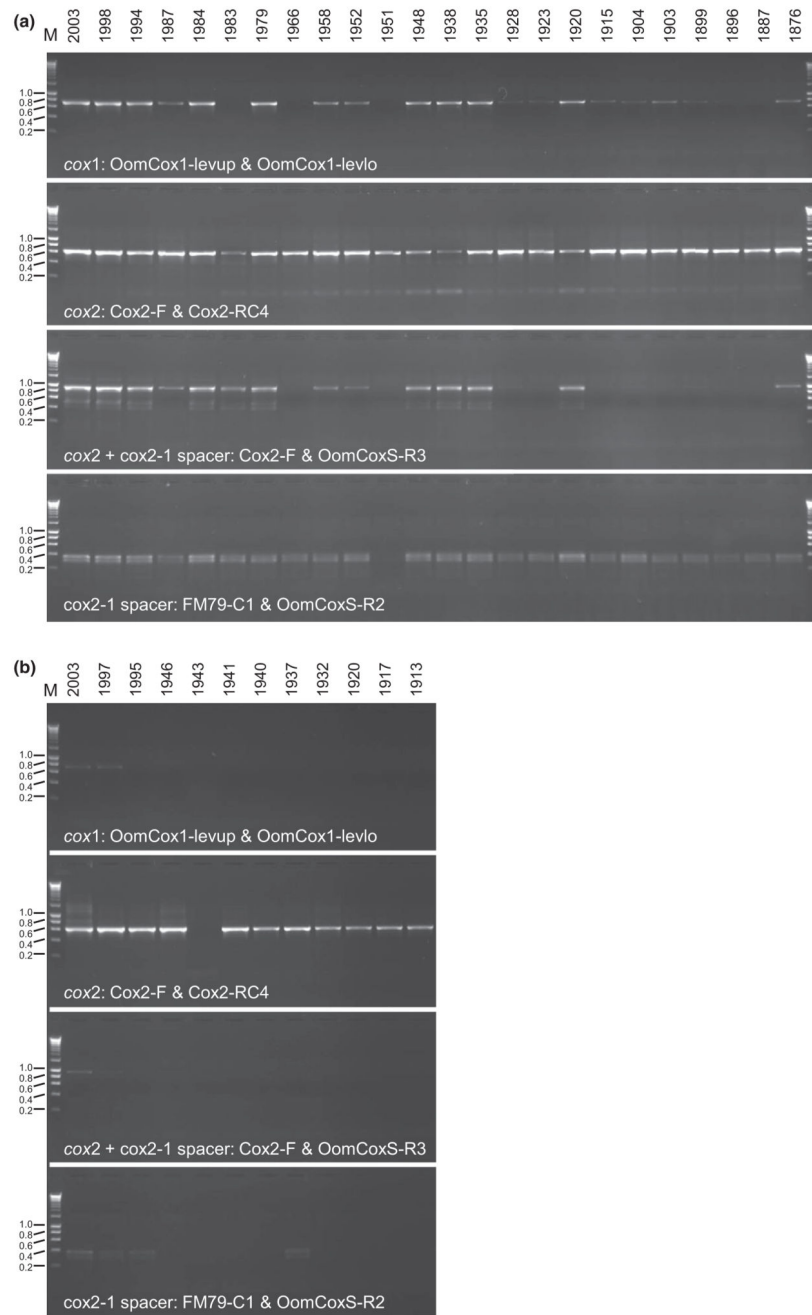


**Fig. 1.** Gene organization of the mitochondrial *cox2-1* gene cluster and location of PCR primers. Arrows above or below the fragment indicate the primers that has been used for amplification.

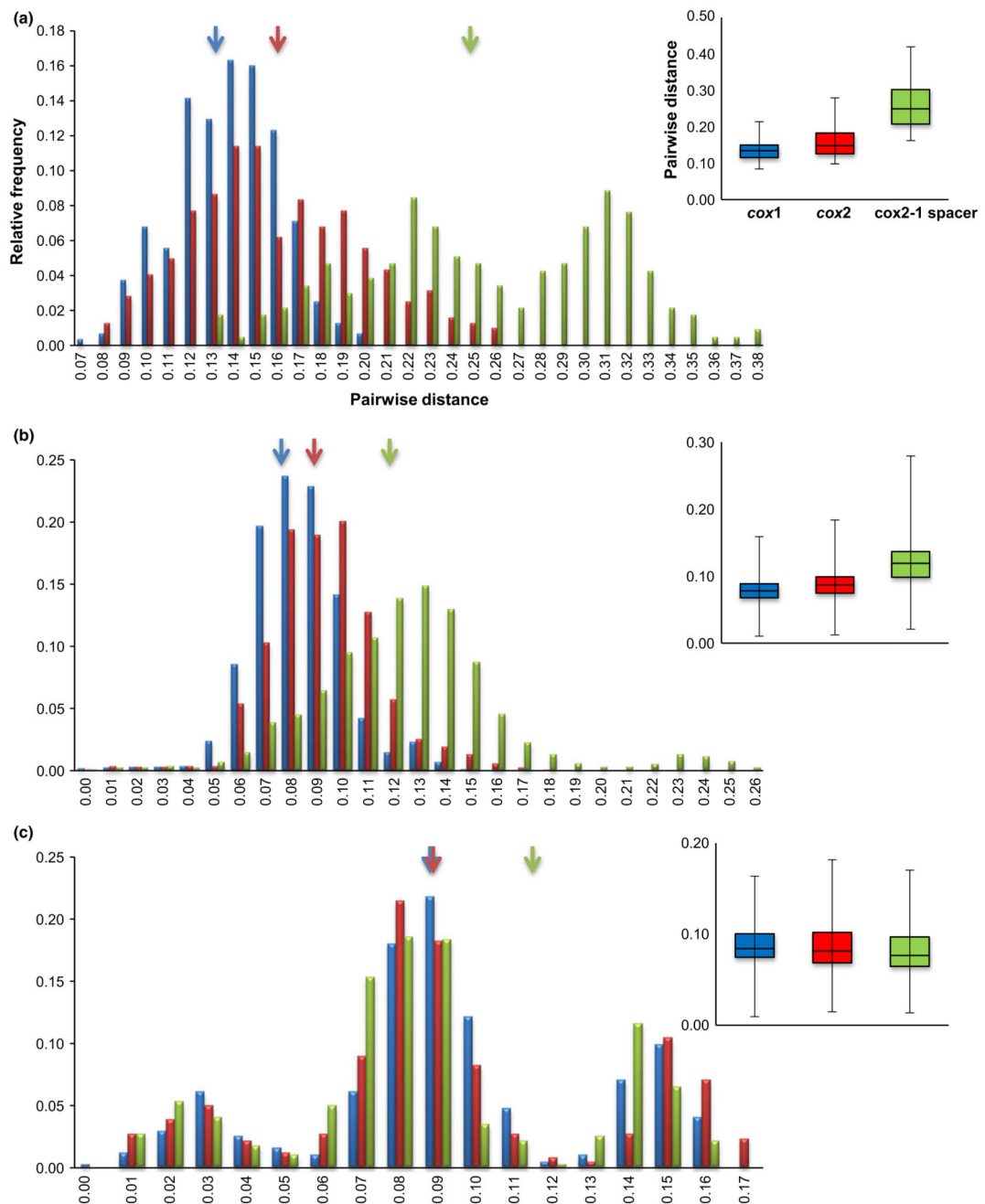


**Fig. 2.**

Gel electropherograms for the amplification of *cox1*, *cox2*, *cox2* + *cox2-1* spacer and *cox2-1* spacer regions on 31 representative oomycete lineages. DNA HyperLadder I (Bioline) was used as a molecular size marker (kb) (M), and fragment sizes are indicated to the right.

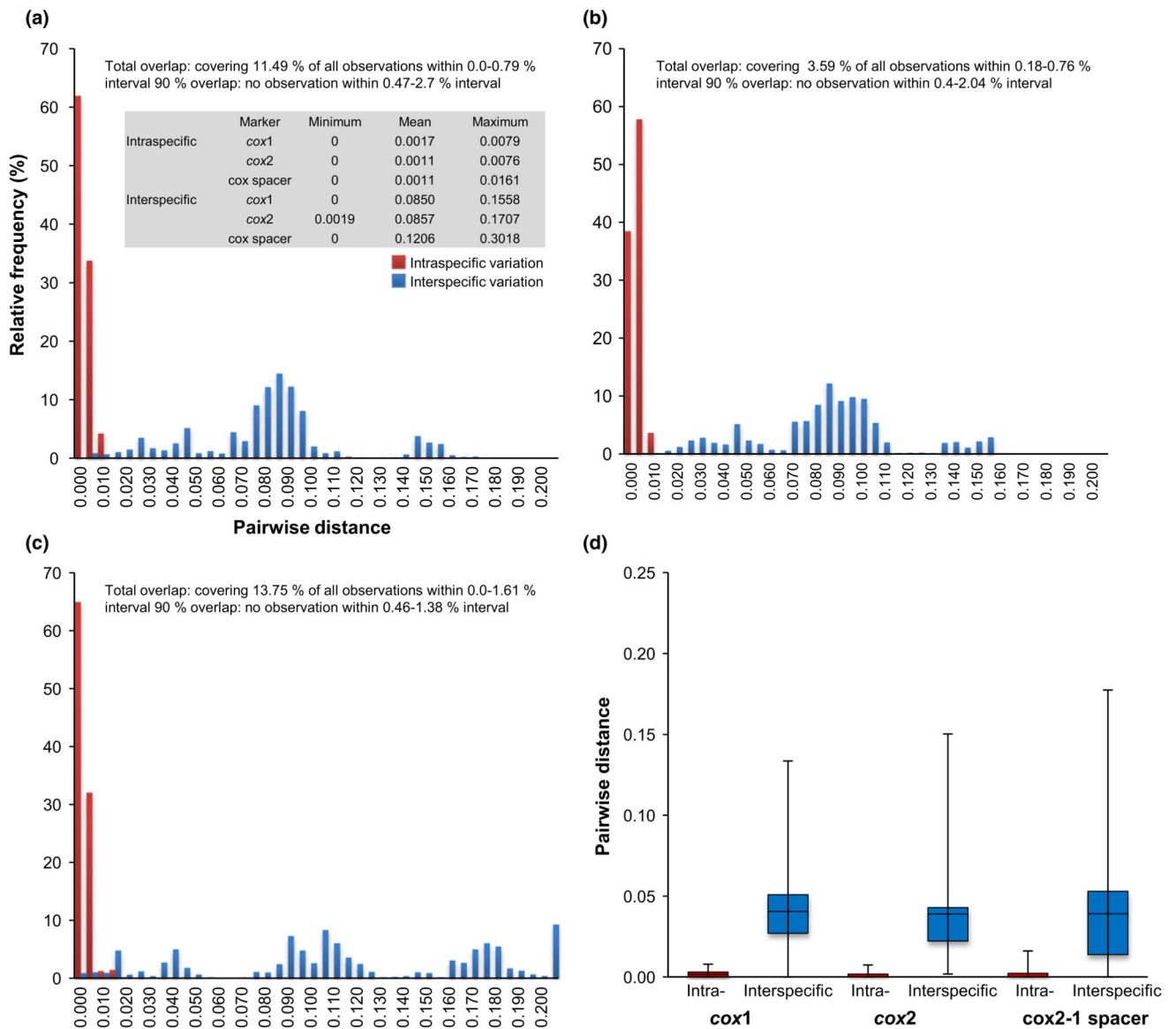


**Fig. 3.** Gel electropherograms for the amplification of *cox1*, *cox2*, *cox2* + *cox2-1* spacer and *cox2-1* spacer regions on herbarium specimens of *Albugo candida* (a) and *Plasmopara viticola* (b). DNA HyperLadder I (Bioline) was used as a molecular size marker (kb) (M), and fragment sizes are indicated to the right.



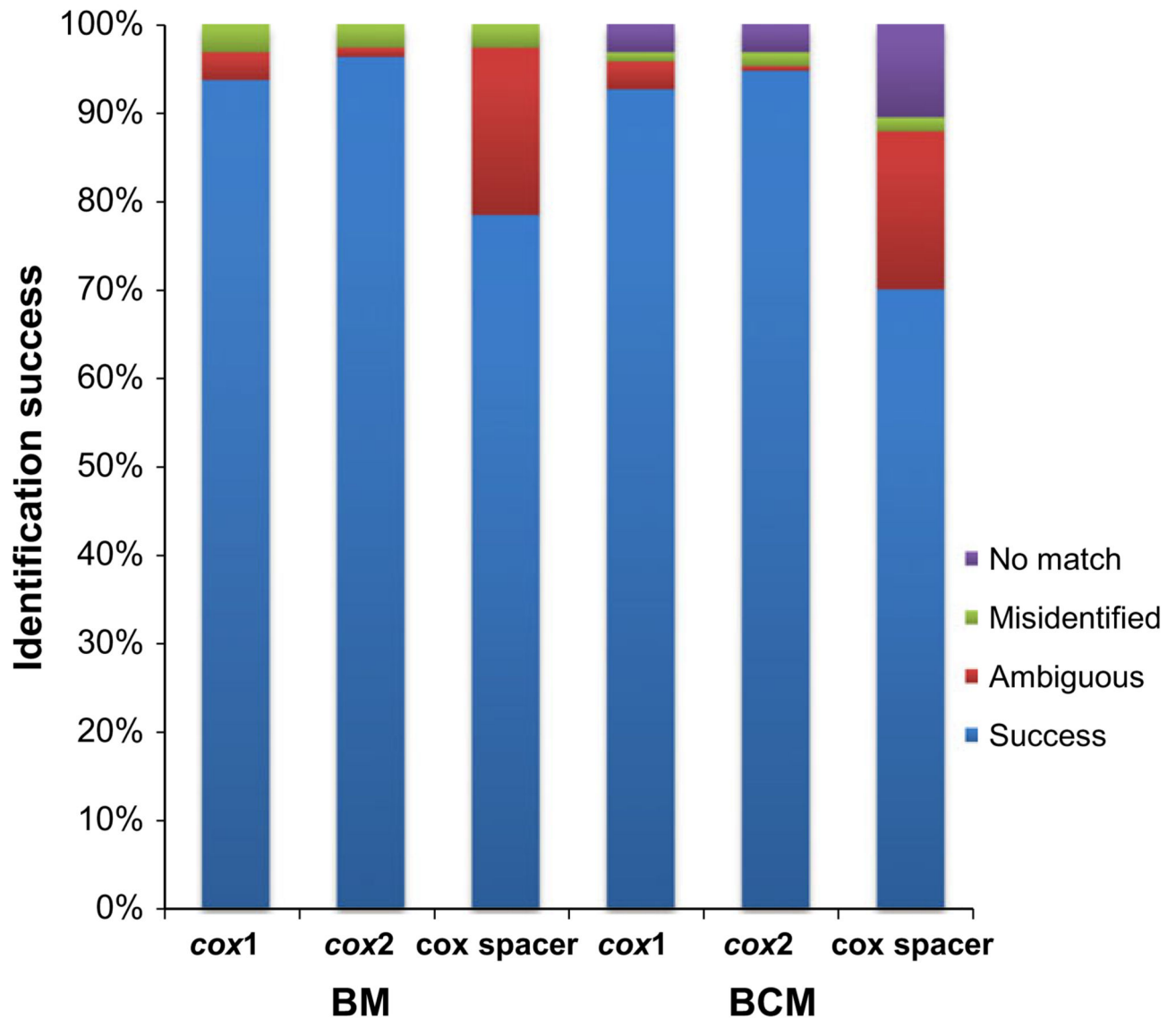
**Fig. 4.**

Distance histograms of the interspecific comparisons of *cox1*, *cox2* and *cox2-1* spacer sequences. Histograms display interspecific variation for 26 genera of Oomycete (a), 110 species of *Peronospora* (b) and 33 species of *Bremia* (c). Arrows point the overall mean pairwise distances for *cox1* (blue), *cox2* (red) and *cox2-1* spacer (green). Box plots depict the variation for intra- and interspecific differences for the three markers. Black bar in the box indicates the median.



**Fig. 5.**

Distance histograms of the intra- and interspecific comparisons of *cox1* (a), *cox2* (b) and *cox2-1 spacer* (c) for *Bremia* species and boxplots (d) depicting the variation for the three markers. A table summarizes distance data. Black bar in the box indicates the median. Total overlap range (with corresponding percentage of observations it represents) and 90% overlap (largest 5% of the intraspecific and lowest 5% of the interspecific excluded) are indicated.



**Fig. 6.** Identification success based on Best Match (BM) and Best Close Match (BCM) criteria for *cox1*, *cox2* and *cox2-1* spacer. Success: correct identifications; Ambiguous: ambiguous identifications; Misidentified: incorrect identifications; and No match: sequences without any match closer than a threshold value below 95% of intraspecific distances.

**Table 1**

Previously published and newly developed primers

<b>Primer</b>	<b>Sequence (5' →3')</b>	<b>Reference</b>
OomCox1-levup	TCAWCWMGATGGCTTTTTCAAC	Robideau <i>et al.</i> (2011)
OomCox1-levlo	CYTCHGGRTGWCCRAAAAACCAAA	Robideau <i>et al.</i> (2011)
FM85mod	RRHWACKTGACTIONATRATACCAAA	Robideau <i>et al.</i> (2011)
FM79-C1	GGNCAATGTAGYGAAATHTG	This study
FM80	AATATCTTTATGATTTGTTGAAA	Martin & Tooley (2003a)
FMPhy-10b	GCAAAAAGCACTAAAAATTAATATAA	Martin <i>et al.</i> (2004)
OomCoxS-R2	GTWGAAAAAABCCAHCCKDKWTGACC	This study
OomCoxS-R3	GTWGAAAAAABCCAHCCKDKWTGAC	This study
Cox2-F	GGCAAATGGGTTTTCAAGATCC	Hudspeth <i>et al.</i> (2000)
Cox2-R	CCATGATTAATACCACAAATTCACTAC	Hudspeth <i>et al.</i> (2000)
Cox2-RC4	TGATTWAYNCCACAAATTCRCTACATTG	This study

**Table 2**

Combinations of primers used for PCR amplification

Target gene	Forward primer	Reverse primer	T (°C) <sup>‡</sup>
<i>cox1</i>	OomCox1-levup	OomCox1-levlo	50, 55
		FM85mod	50, 55
<i>cox2</i>	Cox2-F	Cox2-R	50, 55
		Cox2-RC4	50
<i>cox2</i> -1 spacer <sup>†</sup>	FM79-C1	FM80	52
		FMPhy-10b	52
		OomCoxS-R2	52
<i>cox2</i> + <i>cox2</i> -1 spacer	Cox2-F	FMPhy-10b	54
		OomCoxS-R3	54

<sup>†</sup>The spacer region between *cox2* and *cox1*.

<sup>‡</sup>Annealing temperature during amplification.

**Table 3**

Summary statistics and interspecific divergences of oomycete taxa represented with one sample

Oomycetes	Gene	Size (bp)	L <sup>†</sup> (bp)	K <sup>‡</sup>	Par <sup>§</sup>	Pi <sup>¶</sup> ± SD	Distinguishable taxa	Overall mean pairwise distance
26 genera	<i>cox1</i>	641	641	83.756	263	0.13067 ± 0.0000122	26	0.1331
	<i>cox2</i>	605	572	89.022	291	0.15563 ± 0.000405	26	0.1586
	cox spacer <sup>**</sup>	260–420	208	46.290	198	0.22255 ± 0.08745	26	0.2500
110 species of <i>Peronospora</i>	<i>cox1</i>	680	660	56.634	278	0.08581 ± 0.010	106	0.0786
	<i>cox2</i>	577	546	52.360	268	0.09590 ± 0.013	108	0.0877
	cox spacer	340–415	226	24.504	148	0.10843 ± 0.05909	106	0.1197
33 species of <i>Bremia</i>	<i>cox1</i>	629	629	60.810	149	0.09668 ± 0.03641	32	0.0881
	<i>cox2</i>	539	539	52.261	171	0.09696 ± 0.03804	33	0.0890
	cox spacer	355–400	434	50.743	141	0.11692 ± 0.05239	29	0.1153

<sup>†</sup>Number of sites compared after removing gaps and misaligned regions.

<sup>‡</sup>Average number of nucleotide difference.

<sup>§</sup>Number of parsimony informative sites.

<sup>¶</sup>Nucleotide diversity.

<sup>\*\*</sup>A spacer region between *cox2* and *cox1*.