The genus Alternaria includes numerous phytopathogenic species, many of which are economically relevant. Traditionally, identification has been based on morphology, but is often hampered by the tendency of some strains to become sterile in culture and by the existence of species-complexes of morphologically similar taxa. This study aimed to assess if strains of four closely-related plant pathogens, i.e., accurately Alternaria dauci (ten strains), Alternaria porri (six), Alternaria solani (ten), and Alternaria tomatophila (ten) could be identified using multilocus phylogenetic analysis and Matrix-Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) profiling of proteins. Phylogenetic analyses were performed on three loci, i.e., the internal transcribed spacer (ITS) region of rRNA, and the glyceraldehyde-3-phosphate dehydrogenase (gpd) and Alternaria major antigen (Alt a 1) genes. Phylogenetic trees based on ITS sequences did not differentiate strains of A. solani, A. tomatophila, and A. porri, but these three species formed a clade separate from strains of A. dauci. The resolution improved in trees based on gpd and Alt a 1, which distinguished strains of the four species as separate clades. However, none provided significant bootstrap support for all four species, which could only be achieved when results for the three loci were combined. MALDI-TOF-based dendrograms showed three major clusters. The first comprised all A. dauci strains, the second included five strains of A. porri and one of A. solani, and the third included all strains of A. tomatophila, as well as all but one strain of A. solani, and one strain of A. porri. Thus, this study shows the usefulness of MALDI-TOF mass spectrometry as a promising tool for identification of these four species of Alternaria which are closely-related plant pathogens.